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<b>(21) International Application Number:</b> PCT/US99/23385 <b>(22) International Filing Date:</b> 06 October 1999 (06.10.1999) <b>(30) Priority Data:</b> 60/103,441 07 October 1998 (07.10.1998) US <b>(60) Parent Application or Grant</b> MAXYGEN, INC. [/]; O. SUBRAMANIAN, Venkiteswaran [/]; O. SUBRAMANIAN, Venkiteswaran [/]; O. QUINE, Jonathan, Alan ; ().		<b>Published</b>
<b>(54) Title: DNA SHUFFLING TO PRODUCE NUCLEIC ACIDS FOR MYCOTOXIN DETOXIFICATION</b> <b>(54) Titre: REARRANGEMENT D'ADN POUR PRODUIRE DES ACIDES NUCLEIQUES DE DETOXICATION CONTRE LES MYCOTOXINES</b>		
<b>(57) Abstract</b>  Methods of shuffling nucleic acids to acquire or enhance mycotoxin detoxification activity, libraries of shuffled mycotoxin detoxification nucleic acids, transgenic cells, plants and DNA shuffling mixtures are provided.		
<b>(57) Abrégé</b>  La présente invention concerne des procédés de réarrangement d'acides nucléiques permettant d'obtenir ou d'augmenter l'activité de détoxification contre les mycotoxines, des bibliothèques d'acide nucléiques de détoxification contre les mycotoxines, des cellules transgéniques, ainsi que des mélanges de réarrangement d'ADN et de plantes.		

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(54) Title: DNA SHUFFLING TO PRODUCE NUCLEIC ACIDS FOR MYCOTOXIN DETOXIFICATION (54) Titre: REARRANGEMENT D'ADN POUR PRODUIRE DES ACIDES NUCLEIQUES DE DETOXICATION CONTRE LES MYCOTOXINES		

(57) Abstract

Methods of shuffling nucleic acids to acquire or enhance mycotoxin detoxification activity, libraries of shuffled mycotoxin detoxification nucleic acids, transgenic cells, plants and DNA shuffling mixtures are provided.

(57) Abrégé

La présente invention concerne des procédés de réarrangement d'acides nucléiques permettant d'obtenir ou d'augmenter l'activité de détoxification contre les mycotoxines, des bibliothèques d'acide nucléiques de détoxification contre les mycotoxines, des cellules transgéniques, ainsi que des mélanges de réarrangement d'ADN et de plantes.

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<b>(54) Title:</b> DNA SHUFFLING TO PRODUCE NUCLEIC ACIDS FOR MYCOTOXIN DETOXIFICATION		
<b>(57) Abstract</b>  Methods of shuffling nucleic acids to acquire or enhance mycotoxin detoxification activity, libraries of shuffled mycotoxin detoxification nucleic acids, transgenic cells, plants and DNA shuffling mixtures are provided.		

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## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/23385

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12N15/10 C12N15/53 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 23758 A (INCYTE PHARMA INC ;GOLI SURYA K (US); HILLMAN JENNIFER L (US)) 4 June 1998 (1998-06-04) page 2, line 7 - line 12 page 2, line 24 - line 29 page 16, line 28 -page 17, line 6 page 25, line 6 - line 9	1-21, 27-32
X	WO 96 06175 A (PIONEER HI BRED INT) 29 February 1996 (1996-02-29) the whole document --- -/--	9,22-26

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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International Application No.

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DUVICK, J., ET AL.: "Detoxification of mycotoxins in plants as a strategy for improving grain quality and disease resistance: Identification of fumonisin-degrading microbes from maize" MOLECULAR GENETICS OF HOST-SPECIFIC TOXINS IN PLANT DISEASE. PROCEEDINGS OF THE 3RD TOTTORI INTERNATIONAL SYMPOSIUM DAISEN, TOTTORI, JAPAN 24-29 AUGUST 1997(1998). KLUWER ACADEMIC PRESS, DORDRECHT, pages 369-381, XP002121275 the whole document</p>	1-32
A	<p>WO 96 12414 A (ERBER ERICH KG) 2 May 1996 (1996-05-02) the whole document</p>	1-32
A	<p>KIMURA M ET AL: "Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of Tr1101" JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 273, no. 3, 16 January 1998 (1998-01-16), pages 1654-1661-1661, XP002125091 ISSN: 0021-9258 the whole document</p>	1-32
A	<p>WO 97 35966 A (MAXYGEN INC ;MINSHULL JEREMY (US); STEMMER WILLEM P C (US)) 2 October 1997 (1997-10-02) the whole document</p>	1-32
A	<p>WO 97 20078 A (AFFYMAX TECH NV ;CRAMERI ANDREAS (US); STEMMER WILLEM P C (US)) 5 June 1997 (1997-06-05) the whole document</p>	1-32
A	<p>HARAYAMA S: "Artificial evolution by DNA shuffling" TRENDS IN BIOTECHNOLOGY, GB, ELSEVIER PUBLICATIONS, CAMBRIDGE, vol. 16, no. 2, February 1998 (1998-02), pages 76-82, XP004107046 ISSN: 0167-7799 the whole document</p>	1-32
	-/-	

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/23385

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CRAMERI A ET AL: "Molecular evolution of an arsenate detoxification pathway y DNA shuffling" NATURE BIOTECHNOLOGY, May 1997 (1997-05), XP002082183 the whole document	1-32
A	WO 98 31816 A (MINSHULL JEREMY S ;SOUZA MERVYN L DE (US); UNIV MINNESOTA (US); SA) 23 July 1998 (1998-07-23) the whole document	1-32
A	WO 98 31837 A (DELCARDAYRE STEPHEN B ;MAXYGEN INC (US); MINSHULL JEREMY (US); NES) 23 July 1998 (1998-07-23) the whole document	13,14, 29-32
A	POMPON D ET AL: "PROTEIN ENGINEERING BY CDNA RECOMBINATION IN YEASTS: SHUFFLING OF MAMMALIAN CYTOCHROME P-450 FUNCTIONS" GENE,NL,ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, vol. 83, no. 1, 15 November 1989 (1989-11-15), pages 15-24, XP000054542 ISSN: 0378-1119 the whole document	11
A	SHINOJI M ET AL: "DESIGN OF A NOVEL P450: A FUNCTIONAL BACTERIAL-HUMAN CYTOCHROME P450 CHIMERA" BIOCHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. EASTON, PA, vol. 37, no. 25, 1998, pages 8848-8852, XP002913528 ISSN: 0006-2960 the whole document	11
A	WO 98 27230 A (MAXYGEN INC ;PATTEN PHILLIP A (US); STEMMER WILLEM P C (US)) 25 June 1998 (1998-06-25) the whole document	12
A	CRAMERI A ET AL: "DNA SHUFFLING OF A FAMILY OF GENES FROM DIVERSE SPECIES ACCELERATES DIRECTED EVOLUTION" NATURE,GB,MACMILLAN JOURNALS LTD. LONDON, vol. 391, 15 January 1998 (1998-01-15), pages 288-291, XP000775869 ISSN: 0028-0836 the whole document	20
-/-		

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/23385

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 00 04158 A (DUVICK JONATHAN P ;PIONEER HI BRED INT (US); GILLIAM JACOB (US); M) 27 January 2000 (2000-01-27) page 12, line 21 -page 13, line 5	1-4,9
E	WO 00 04160 A (PIONEER HI BRED INT ;FOLKERTS OTTO (US); CRASTA OSWALD R (US); CUR) 27 January 2000 (2000-01-27) page 26, line 25 -page 27, line 18	1-4,9



# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 99/23385

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9823758 A	04-06-1998	US 5817497 A AU 5181198 A US 5976528 A	06-10-1998 22-06-1998 02-11-1999
WO 9606175 A	29-02-1996	US 5792931 A AU 697831 B AU 3491095 A AU 9725398 A CA 2195784 A EP 0775211 A EP 0981953 A NZ 292668 A US 5716820 A US 6025188 A ZA 9506531 A	11-08-1998 15-10-1998 14-03-1996 15-04-1999 29-02-1996 28-05-1997 01-03-2000 25-11-1998 10-02-1998 15-02-2000 25-03-1996
WO 9612414 A	02-05-1996	AT 504 U AT 175073 T AU 3736595 A BR 9509399 A CA 2192983 A CN 1158554 A CZ 9603683 A DE 59504703 D EP 0786945 A ES 2128086 T HR 950526 A HU 76374 A IL 115650 A JP 2983639 B JP 9511657 T PL 319755 A SK 167896 A ZA 9508786 A	27-12-1995 15-01-1999 15-05-1996 04-11-1997 02-05-1996 03-09-1997 11-06-1997 11-02-1999 06-08-1997 01-05-1999 30-04-1997 28-08-1997 14-07-1999 29-11-1999 25-11-1997 18-08-1997 04-06-1997 13-05-1996
WO 9735966 A	02-10-1997	US 5837458 A AU 2337797 A AU 2542697 A CA 2247930 A EP 0932670 A EP 0906418 A WO 9735957 A AU 713952 B AU 1087397 A CA 2239099 A EP 0876509 A EP 0911396 A JP 2000500981 T WO 9720078 A	17-11-1998 17-10-1997 17-10-1997 02-10-1997 04-08-1999 07-04-1999 02-10-1997 16-12-1999 19-06-1997 05-06-1997 11-11-1998 28-04-1999 02-02-2000 05-06-1997
WO 9720078 A	05-06-1997	US 5811238 A AU 713952 B AU 1087397 A AU 2542697 A CA 2239099 A EP 0876509 A EP 0906418 A EP 0911396 A	22-09-1998 16-12-1999 19-06-1997 17-10-1997 05-06-1997 11-11-1998 07-04-1999 28-04-1999

# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9720078 A		JP 2000500981 T	02-02-2000
		WO 9735966 A	02-10-1997
		US 5837458 A	17-11-1998
WO 9831816 A	23-07-1998	AU 6029998 A	07-08-1998
WO 9831837 A	23-07-1998	AU 5920998 A	07-08-1998
WO 9827230 A	25-06-1998	AU 5729298 A	15-07-1998
		EP 0946755 A	06-10-1999
WO 0004158 A	27-01-2000	NONE	
WO 0004160 A	27-01-2000	WO 0004159 A	27-01-2000



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(21) International Application Number: <b>PCT/US99/23385</b>		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GR, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
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(71) Applicant (for all designated States except US): MAXYGEN, INC. [US/US]; 515 Galveston Drive, Redwood City, CA 94063 (US).			
(72) Inventor; and (75) Inventor/Applicant (for US only): SUBRAMANIAN, Venkiteswaran [US/US]; 3980 Corte Mar de Hierba, San Diego, CA 92130 (US).		Published Without international search report and to be republished upon receipt of that report.	
(74) Agents: QUINE, Jonathan, Alan; The Law Offices of Jonathan Alan Quine, P.O. Box 458, Alameda, CA 94501 (US) et al.			
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# DNA SHUFFLING TO PRODUCE NUCLEIC ACIDS FOR MYCOTOXIN DETOXIFICATION

## CROSS REFERENCE TO RELATED APPLICATIONS

The present application is a non-provisional of "DNA SHUFFLING TO PRODUCE NUCLEIC ACIDS FOR MYCOTOXIN DETOXIFICATION" by Subramanian, USSN 60/103,441, filed October 7, 1998.

## FIELD OF THE INVENTION

This invention pertains to the shuffling of nucleic acids to achieve or enhance mycotoxin detoxification, especially in plants.

## BACKGROUND OF THE INVENTION

"Mycotoxins" generically refer to a number of toxic molecules produced by fungal species, such as polyketides (including aflatoxins, demethylsterigmatocystin, O-methylsterigmatocystin etc.), fumonisins, alperisins (e.g., A<sub>1</sub>, A<sub>2</sub>, B<sub>1</sub>, B<sub>2</sub>), sphingofungins (A, B, C and D), trichothecenes, fumifungins, and the like. Polyketides are a large structurally diverse class of secondary metabolites synthesized by bacteria, fungi, and plants and are formed by a polyketide synthase (PKS) through the sequential condensation of small carboxylic acids. Katz and Donandio (1993) Annu Rev. Microbiol. 47:875-912; Brown et al. (1996) PNAS 93:14873-14877; Silva et al. (1996) J. Biol Chem. 271: 13600-608.

Aflatoxin B<sub>1</sub> is the principal member of the aflatoxin (AF) family of polyketide mycotoxins produced by *Aspergillus parasiticus*, *Aspergillus flavus* and *Aspergillus nomius*. Aflatoxin B<sub>1</sub> is the most potent mycotoxin known to man. For example, AF was characterized as the causative agent for the death of more than a hundred thousand poultry in England that had ingested AF-contaminated peanut meal. This discovery led to legislation regulating the trade of AF-contaminated agricultural commodities.

Sterigmatocystin (ST) is a related polyketide mycotoxin, which is produced by several members of the *Aspergillus*. ST is the second to last intermediate in

the biosynthesis of AF. Kelkar et al. (1997) J. Biol Chem. 272: 1589-94. Various *Aspergillus* species that produce AF and ST are known to be pathogenic to corn, grains and nuts and are known to produce these mycotoxins during the growth of the crops and during storage, leading to the introduction of AF and ST into primary food stuffs. AF and ST are acutely toxic and carcinogenic and are a serious concern from human and animal health perspective. Busby & Wogan (1985) in Chemical Carcinogens (Searle ed., 1985) pp 945-1136, American Chemical Society, Washington D.C.

Trichothecenes are another family of sesquiterpenoid mycotoxins produced by *Fusarium* species and other molds that are known plant pathogens. These compounds are potent inhibitors of protein synthesis in eukaryotes (Kimura et al. (1998) J. Biol Chem. 273: 1654-1661) and reportedly bind to the 60S ribosomal subunits to prevent polypeptide chain initiation or elongation. Trichothecenes are also an important group of mycotoxins that cause serious problems of food pollution. They have been implicated in incidents of mycotoxicosis including vomiting, dermatitis and hemorrhagic septicemia in humans and livestock, resulting in loss of productivity and even death. Lastly, fumonisins (F) are another structurally distinct class of mycotoxins produced by several *Fusarium* species that is involved in food poisoning and toxic effects. Scott (1993) International Journal of Food Microbiology 18:257-270 and the references therein provide a review of the Fumonisins.

Thus, the contamination of corn, grains and nuts with various types of mycotoxins produced by pathogenic species such as *Aspergillus* and *Fusarium* is a major health and food pollution problem, as well as causing reduction in crop yields by being toxic to infected plants. These mycotoxins survive food processing, which adds to the problem. It is well known that ST and AF induce liver cancer and are linked to a specific mutation in a tumor suppressor gene. Brown et al. (1996) PNAS 93: 14873-14877. Natural aflatoxins and other mycotoxins like ST do not pose a major health threat *per se*; however, renal and hepatic oxidative detoxification of these compounds in contaminated foods by cytochrome P450 enzymes yields an epoxide which is cytotoxic.

For example, AFB1 is converted to its 15,16-exo-epoxide, which is a highly toxic mutagen. Silva et al. (1996), *supra* and references therein. It has been shown that this epoxide targets guanine residues and selectively alkylates the N-7 position

5 of this purine in double-stranded DNA. Depurination of the alkylated base has been  
correlated to bladder cancer in laboratory mice, teratogenic effects in chicken embryos  
and liver cancer in humans. A direct correlation between DNA damage and human  
10 cancer has been established and is related to the mutational hot spots of p53, an important  
5 tumor suppressor gene. Approximately 50% of all cancers have associated altered p53  
sequences.

Trading of AF-contaminated agricultural commodities is tightly regulated  
15 at both national and international levels. Compliance to these regulations causes the loss  
of millions of dollars in agricultural produce in US each year. Trade sanctions and  
10 health effects on mycotoxin contaminated grains add significantly to the losses (Brown et  
al. (1996) PNAS 93: 14873-14877).

Accordingly, it is highly desirable to transform various mycotoxins  
produced by fungal pathogens in various crops into inactive compounds with respect to  
plant, human and animal toxicity. This would alleviate important food pollution  
25 problems, as well as cost associated with complying with detecting AF-contamination in  
15 various crop commodities and destroying them. Surprisingly, the present invention  
provides for the detoxification of mycotoxin by transformation of the mycotoxins into  
30 non-toxic compounds. This detoxification is particularly useful in crops, thereby solving  
each of the problems outlined above, as well as providing a variety of other features  
20 which will be apparent upon review.

#### SUMMARY OF THE INVENTION

35 In the present invention, DNA shuffling is used to generate new or  
improved mycotoxin detoxification genes. These mycotoxin detoxification genes are  
used to provide enzymes which degrade mycotoxins, in agricultural and industrial  
40 25 processes. These new and/or improved genes have surprisingly superior properties as  
compared to naturally occurring mycotoxin detoxification genes.

In the methods for obtaining mycotoxin resistant genes, a plurality of  
parental forms (homologs) of a selected nucleic acid are recombined. The selected  
45 nucleic acid is derived either from one or more parental nucleic acid(s) which encodes an  
30 enzyme which degrades or modifies a mycotoxin, or a fragment thereof, or from a  
parental nucleic acid which does not encode mycotoxin detoxification, but which is a



5 substrate for DNA shuffling to develop monooxygenase activity. The plurality of forms  
of the selected nucleic acid differ from each other in at least one (and typically two or  
more) nucleotides, and, upon recombination, provide a library of recombinant mycotoxin  
10 detoxification nucleic acids. The library can be an *in vitro* set of molecules, or present in  
5 cells, phage or the like. The library is screened to identify at least one recombinant  
mycotoxin detoxification nucleic acid that exhibits distinct or improved mycotoxin  
detoxification activity (typically in an encoded polypeptide) compared to the parental  
15 nucleic acid or nucleic acids.

In selecting for mycotoxin detoxification activity, a candidate shuffled  
10 DNA can be tested for encoded mycotoxin detoxification activity in essentially any  
process. Common processes that can be screened include screening for inactivation or  
20 modification of an aflatoxin, inactivation or modification of a sterigmatocystin,  
inactivation or modification of a trichothecene, and inactivation or modification of a  
fumonisin. Similarly, instead of, or in addition to, testing for an increase in mycotoxin  
25 detoxification activity, it is also desirable to screen for shuffled nucleic acids which  
produce higher levels of a mycotoxin detoxification nucleic acid or enhanced or reduced  
recombinant mycotoxin detoxification polypeptide expression, or increased stability  
30 encoded by the recombinant mycotoxin resistant nucleic acid.

A variety of screening methods can be used to screen a library, depending  
20 on the mycotoxin detoxification activity for which the library is selected. By way of  
example, the library to be screened can be present in a population of cells. The library is  
35 selected by growing the cells in or on a medium comprising the mycotoxin to be  
degraded and selecting for a detected physical difference between, e.g., oxidized or  
reduced forms of the mycotoxin and the non-oxidized or reduced form of the mycotoxin,  
40 25 either in the cell, or the extracellular medium. Alternately, survival of library cells on a  
medium which includes a mycotoxin can be used to screen the library.

Iterative selection for mycotoxin detoxification nucleic acids is also a  
45 feature of the invention. In these methods, a selected nucleic acid identified as encoding  
mycotoxin detoxification activity can be shuffled, either with the parental nucleic acids,  
30 or with other nucleic acids (e.g., mutated forms of the selected nucleic acid) to produce a  
second shuffled library. The second shuffled library is then selected for one or more

5 form of mycotoxin detoxification activity, which can be the same or different than the mycotoxin detoxification activity previously selected.

10 This process can be iteratively repeated as many times as desired, until a nucleic acid with optimized or desired mycotoxin detoxification properties is obtained. If  
5 desired, any nucleic acid identified by any of the methods herein can be cloned and, optionally, expressed. Because of the need to reduce mycotoxin pollution/contamination of  
15 foods, it is desirable to express mycotoxin detoxification nucleic acids in, e.g., plants, thereby reducing the occurrence of mycotoxins in the plants. Furthermore, mycotoxin detoxification in plants also adds to the vigor of the plants.

10 The invention also provides methods of increasing mycotoxin detoxification activity by whole genome shuffling. In these methods, a plurality of  
20 genomic nucleic acids are shuffled in a cell (in whole cell shuffling, entire genomes are shuffled, rather than specific sequences, although "spiking" of selected nucleic acids can be used to bias shuffling outcomes). The resulting shuffled nucleic acids are selected for  
25 one or more mycotoxin detoxification traits. The genomic nucleic acids can be from a species or strain different from the cell in which activity is desired. Similarly, the shuffling reaction can be performed in cells using genomic DNA from the same or  
30 different species, or strains. Strains or enzymes exhibiting enhanced activity can be identified.

20 The distinct or improved activity encoded by a nucleic acid identified after shuffling can encode one or more of a variety of properties, including, e.g., inactivation  
35 or modification of a polyketide, an aflatoxin, inactivation or modification of a sterigmatocystin, inactivation or modification of a trichothecene, inactivation or modification of a fumonisin, an increased ability to chemically modify a mycotoxin, an  
40 25 increase in the range of mycotoxin substrates which the distinct or improved nucleic acid operates on, an increased expression level of a polypeptide encoded by the nucleic acid, a decrease in susceptibility of a polypeptide encoded by the nucleic acid to protease  
45 cleavage, a decrease in susceptibility of a polypeptide encoded by the nucleic acid to high or low pH levels, a decrease in susceptibility of the protein encoded by the nucleic acid to  
30 high or low temperatures, and a decrease in toxicity to a host cell of a polypeptide encoded by the selected nucleic acid.

5 The selected nucleic acids to be shuffled can be from any of a variety of  
sources, including synthetic or cloned DNAs. Exemplar targets for recombination  
include: nucleic acids encoding a monooxygenase, a P450, trichothecene-3-O-  
10 acetyltransferase, a 3-O-Methyltransferase, a glutathione S-transferase, an epoxide  
5 hydrolase, an isomerase, a macrolide-O-acetyltransferase, a 3-O-acetyltransferase, and a  
cis-diol producing monooxygenase which is specific for furan. Typically, shuffled  
nucleic acids are cloned into expression vectors to achieve desired expression levels.

15 One feature of the invention is the production of libraries and shuffling  
mixtures for use in the methods as set forth above. For example, a phage display library  
10 comprising shuffled forms of a nucleic acid is provided. Similarly, a shuffling mixture  
comprising at least three homologous DNAs, each of which is derived from a nucleic  
20 acid encoding a polypeptide or polypeptide fragment, is provided. These polypeptides  
can be, for example, any of those noted herein.

25 Isolated nucleic acids identified by selection of the libraries in the methods  
15 above are also a feature of the invention, as are kits comprising any of: mycotoxin  
detoxification nucleic acid libraries, shuffled mycotoxin detoxification nucleic acids,  
instructional materials for practicing any of the methods herein, containers for holding  
30 other kit components, and the like.

#### BRIEF DESCRIPTION OF THE FIGURES

20 Not Applicable.

#### DEFINITIONS

35 Unless clearly indicated to the contrary, the following definitions  
supplement definitions of terms known in the art.

40 A "recombinant monooxygenase nucleic acid" is a recombinant nucleic  
25 acid encoding a protein or RNA which confers monooxygenase activity to a cell when the  
nucleic acid is expressed in the cell.

45 A "recombinant" nucleic acid is a nucleic acid produced by recombination  
between two or more nucleic acids, or any nucleic acid made by an *in vitro* or artificial  
process. The term "recombinant" when used with reference to a cell indicates that the  
30 cell comprises (and optionally replicates) a heterologous nucleic acid, or expresses a  
peptide or protein encoded by a heterologous nucleic acid. Recombinant cells can

5 contain genes that are not found within the native (non-recombinant) form of the cell.  
Recombinant cells can also contain genes found in the native form of the cell where the  
genes are modified and re-introduced into the cell by artificial means. The term also  
10 encompasses cells that contain a nucleic acid endogenous to the cell that has been  
artificially modified without removing the nucleic acid from the cell; such modifications  
5 include those obtained by gene replacement, site-specific mutation, and related  
techniques.

15 A "recombinant mycotoxin detoxification nucleic acid" is a recombinant  
nucleic acid encoding a protein or RNA which confers mycotoxin detoxification or  
10 degradation activity to a cell when the nucleic acid is expressed in the cell (and, most  
typically, translated into a polypeptide).

20 A "plurality of forms" of a selected nucleic acid refers to a plurality of  
homologs of the nucleic acid. The homologs can be from naturally occurring homologs  
(e.g., two or more homologous genes) or by artificial synthesis of one or more nucleic  
25 acids having related sequences, or by modification of one or more nucleic acid to  
produce related nucleic acids. Nucleic acids are homologous when they are derived,  
naturally or artificially, from a common ancestor sequence. During natural evolution,  
30 this occurs when two or more descendent sequences diverge from a parent sequence over  
time, i.e., due to mutation and natural selection. Under artificial conditions, divergence  
occurs, e.g., in one of two ways. First, a given sequence can be artificially recombined  
20 with another sequence, as occurs, e.g., during typical cloning, to produce a descendent  
nucleic acid. Alternatively, a nucleic acid can be synthesized *de novo*, by synthesizing a  
35 nucleic acid which varies in sequence from a given parental nucleic acid sequence.

40 When there is no explicit knowledge about the ancestry of two nucleic  
acids, homology is typically inferred by sequence comparison between two sequences.  
25 Where two nucleic acid sequences show sequence similarity it is inferred that the two  
nucleic acids share a common ancestor. The precise level of sequence similarity required  
to establish homology varies in the art depending on a variety of factors. For purposes of  
45 this disclosure, two sequences are considered homologous where they share sufficient  
sequence identity to allow direct recombination to occur between two nucleic acid  
30 molecules (as opposed to recombination using oligonucleotide intermediates, which does

5 not require sequence similarity to achieve recombination). Typically, nucleic acids  
require regions of close similarity spaced roughly the same distance apart to permit  
10 recombination to occur. The recombination can be in vitro or in vivo.

10 The terms "identical" or percent "identity," in the context of two or more  
5 nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences  
that are the same or have a specified percentage of amino acid residues or nucleotides  
that are the same, when compared and aligned for maximum correspondence, as  
15 measured using one of the sequence comparison algorithms described below (or other  
algorithms available to persons of skill) or by visual inspection.

10 The phrase "substantially identical," in the context of two nucleic acids or  
20 polypeptides (e.g., DNAs encoding a monooxygenase, or the amino acid sequence of the  
monooxygenase) refers to two or more sequences or subsequences that have at least about  
60%, preferably 80%, most preferably 90-95% nucleotide or amino acid residue identity,  
when compared and aligned for maximum correspondence, as measured using one of the  
25 following sequence comparison algorithms or by visual inspection. Such "substantially  
identical" sequences are typically considered to be homologous. Preferably, the  
"substantial identity" exists over a region of the sequences that is at least about 50  
30 residues in length, more preferably over a region of at least about 100 residues, and most  
preferably the sequences are substantially identical over at least about 150 residues, or  
20 over the full length of the two sequences to be compared.

35 For sequence comparison and homology determination, typically one  
sequence acts as a reference sequence to which test sequences are compared. When  
using a sequence comparison algorithm, test and reference sequences are input into a  
computer, subsequence coordinates are designated, if necessary, and sequence algorithm  
40 25 program parameters are designated. The sequence comparison algorithm then calculates  
the percent sequence identity for the test sequence(s) relative to the reference sequence,  
based on the designated program parameters.

45 Optimal alignment of sequences for comparison can be conducted, e.g., by  
the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by  
30 the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443  
(1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad.*

5           *Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (*see generally*, Ausubel *et al.*, *infra*).

10                     5           One example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring  
15                     10           sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then  
20                     15           extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in  
25                     20           each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

30                     30           In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*,

5 Karlin & Altschul (1993) *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787). One measure of  
similarity provided by the BLAST algorithm is the smallest sum probability (P(N)),  
which provides an indication of the probability by which a match between two nucleotide  
10 or amino acid sequences would occur by chance. For example, a nucleic acid is  
5 considered similar to a reference sequence if the smallest sum probability in a comparison  
of the test nucleic acid to the reference nucleic acid is less than about 0.1, more  
preferably less than about 0.01, and most preferably less than about 0.001.

15 Another indication that two nucleic acid sequences are substantially  
identical/ homologous is that the two molecules hybridize to each other under stringent  
10 conditions. The phrase "hybridizing specifically to," refers to the binding, duplexing, or  
hybridizing of a molecule only to a particular nucleotide sequence under stringent  
20 conditions, including when that sequence is present in a complex mixture (*e.g.*, total  
cellular) DNA or RNA. "Bind(s) substantially" refers to complementary hybridization  
between a probe nucleic acid and a target nucleic acid and embraces minor mismatches  
25 that can be accommodated by reducing the stringency of the hybridization media to  
15 achieve the desired detection of the target polynucleotide sequence.

30 "Stringent hybridization conditions" and "stringent hybridization wash  
conditions" in the context of nucleic acid hybridization experiments such as Southern and  
northern hybridizations are sequence dependent, and are different under different  
20 environmental parameters. Longer sequences hybridize specifically at higher  
temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen  
35 (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization  
with Nucleic Acid Probes* part I chapter 2 "Overview of principles of hybridization and  
the strategy of nucleic acid probe assays," Elsevier, New York. Generally, highly  
40 25 stringent hybridization and wash conditions are selected to be about 5° C lower than the  
thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength and pH.  
Typically, under "stringent conditions" a probe will hybridize to its target subsequence,  
but no to unrelated sequences.

45 The  $T_m$  is the temperature (under defined ionic strength and pH) at which  
30 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent  
conditions are selected to be equal to the  $T_m$  for a particular probe. An example of



5 stringent hybridization conditions for hybridization of complementary nucleic acids which  
have more than 100 complementary residues on a filter in a Southern or northern blot is  
10 50% formamide with 1 mg of heparin at 42°C, with the hybridization being carried out  
overnight. An example of highly stringent wash conditions is 0.15M NaCl at 72°C for  
5 about 15 minutes. An example of stringent wash conditions is a 0.2x SSC wash at 65°C  
for 15 minutes (*see*, Sambrook, *infra.*, for a description of SSC buffer). Often, a high  
15 stringency wash is preceded by a low stringency wash to remove background probe  
signal. An example medium stringency wash for a duplex of, *e.g.*, more than 100  
nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency wash for a  
10 duplex of, *e.g.*, more than 100 nucleotides, is 4-6x SSC at 40°C for 15 minutes. For  
short probes (*e.g.*, about 10 to 50 nucleotides), stringent conditions typically involve salt  
20 concentrations of less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion  
concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least  
about 30°C. Stringent conditions can also be achieved with the addition of destabilizing  
25 agents such as formamide. In general, a signal to noise ratio of 2x (or higher) than that  
observed for an unrelated probe in the particular hybridization assay indicates detection  
of a specific hybridization.

30 A further indication that two nucleic acid sequences or polypeptides are  
substantially identical/homologous is that the polypeptide encoded by the first nucleic  
20 acid is immunologically cross reactive with, or specifically binds to, the polypeptide  
encoded by the second nucleic acid. Thus, a polypeptide is typically substantially  
35 identical to a second polypeptide, for example, where the two peptides differ only by  
conservative substitutions.

40 "Conservatively modified variations" of a particular polynucleotide  
25 sequence refers to those polynucleotides that encode identical or essentially identical  
amino acid sequences, or where the polynucleotide does not encode an amino acid  
sequence, to essentially identical sequences. Because of the degeneracy of the genetic  
45 code, a large number of functionally identical nucleic acids encode any given  
polypeptide. For instance, the codons CGU, CGC, CGA, CGG, AGA, and AGG all  
30 encode the amino acid arginine. Thus, at every position where an arginine is specified by  
a codon, the codon can be altered to any of the corresponding codons described without



5 altering the encoded polypeptide. Such nucleic acid variations are "silent variations,"  
which are one species of "conservatively modified variations." Every polynucleotide  
sequence described herein which encodes a polypeptide also describes every possible  
10 silent variation, except where otherwise noted. One of skill will recognize that each  
5 codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine)  
can be modified to yield a functionally identical molecule by standard techniques.  
Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is  
15 implicit in each described sequence.

Furthermore, one of skill will recognize that individual substitutions,  
10 deletions or additions which alter, add or delete a single amino acid or a small percentage  
20 of amino acids (typically less than 5%, more typically less than 1%) in an encoded  
sequence are "conservatively modified variations" where the alterations result in the  
substitution of an amino acid with a chemically similar amino acid. Conservative  
substitution tables providing functionally similar amino acids are well known in the art.  
25 The following five groups each contain amino acids that are conservative substitutions for  
15 one another: Aliphatic: Glycine (G), Alanine (A), Valine (V), Leucine (L), Isoleucine  
(I); Aromatic: Phenylalanine (F), Tyrosine (Y), Tryptophan (W); Sulfur-containing:  
30 Methionine (M), Cysteine (C); Basic: Arginine (R), Lysine (K), Histidine (H); Acidic:  
Aspartic acid (D), Glutamic acid (E), Asparagine (N), Glutamine (Q). *See also*,  
20 Creighton (1984) *Proteins*, W.H. Freeman and Company.

35 In addition, individual substitutions, deletions or additions which alter, add  
or delete a single amino acid or a small percentage of amino acids in an encoded  
sequence are also "conservatively modified variations." Sequences that differ by  
conservative variations are generally homologous.

40 25 A "subsequence" refers to a sequence of nucleic acids or amino acids that  
comprise a part of a longer sequence of nucleic acids or amino acids (*e.g.*, polypeptide)  
respectively.

45 The term "gene" is used broadly to refer to any segment of DNA  
associated with expression of a given RNA or protein. Thus, genes include regions  
30 encoding expressed RNAs (which typically include polypeptide coding sequences) and,  
often, the regulatory sequences required for their expression. Genes can be obtained

5 from a variety of sources, including cloning from a source of interest or synthesizing  
from known or predicted sequence information, and may include sequences designed to  
have desired parameters.

10 The term "isolated", when applied to a nucleic acid or protein, denotes  
5 that the nucleic acid or protein is essentially free of other cellular components with which  
it is associated in the natural state.

15 The term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides  
and polymers thereof in either single- or double-stranded form. Unless specifically  
limited, the term encompasses nucleic acids containing known analogues of natural  
10 nucleotides which have similar binding properties as the reference nucleic acid and are  
metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise  
20 indicated, a particular nucleic acid sequence also implicitly encompasses conservatively  
modified variants thereof (*e.g.* degenerate codon substitutions) and complementary  
sequences and as well as the sequence explicitly indicated. Specifically, degenerate  
25 codon substitutions may be achieved by generating sequences in which the third position  
of one or more selected (or all) codons is substituted with mixed-base and/or  
deoxyinosine residues (Batzer *et al.* (1991) *Nucleic Acid Res.* 19: 5081; Ohtsuka *et al.*  
30 (1985) *J. Biol. Chem.* 260: 2605-2608; Cassol *et al.* (1992); Rossolini *et al.* (1994) *Mol.*  
*Cell. Probes* 8: 91-98). The term nucleic acid is generic to the terms "gene", "DNA,"  
20 "cDNA", "oligonucleotide," "RNA," "mRNA," and the like.

35 "Nucleic acid derived from a gene" refers to a nucleic acid for whose  
synthesis the gene, or a subsequence thereof, has ultimately served as a template. Thus,  
an mRNA, a cDNA reverse transcribed from an mRNA, an RNA transcribed from that  
cDNA, a DNA amplified from the cDNA, an RNA transcribed from the amplified DNA,  
40 25 *etc.*, are all derived from the gene and detection of such derived products is indicative of  
the presence and/or abundance of the original gene and/or gene transcript in a sample.

45 A nucleic acid is "operably linked" when it is placed into a functional  
relationship with another nucleic acid sequence. For instance, a promoter or enhancer is  
operably linked to a coding sequence if it increases the transcription of the coding  
30 sequence.

5 A "recombinant expression cassette" or simply an "expression cassette" is  
a nucleic acid construct, generated recombinantly or synthetically, with nucleic acid  
elements that are capable of effecting expression of a structural gene in hosts compatible  
10 with such sequences. Expression cassettes include at least promoters and optionally,  
5 transcription termination signals. Typically, the recombinant expression cassette includes  
a nucleic acid to be transcribed (*e.g.*, a nucleic acid encoding a desired polypeptide), and  
a promoter. Additional factors necessary or helpful in effecting expression may also be  
15 used as described herein. For example, an expression cassette can also include  
nucleotide sequences that encode a signal sequence that directs secretion of an expressed  
10 protein from the host cell. Transcription termination signals, enhancers, and other  
nucleic acid sequences that influence gene expression, can also be included in an  
20 expression cassette.

#### DETAILED DISCUSSION OF THE INVENTION

25 It is highly desirable to enzymatically transform various mycotoxins  
15 produced by fungal pathogens, into inactive compounds with respect to plant, human and  
animal toxicity. This eliminates reductions in crop yield and subsequent food pollution  
by mycotoxins, as well as costs associated with complying with detecting AF-  
30 contamination in various crop commodities and destroying them.

Polyketides are synthesized in fungi, *e.g.*, by polyketide synthase. The  
20 enzyme facilitates the reiterative condensation of simple carboxylic acids; typically,  
acetyl-CoA as a starter unit and malonyl-CoA serves as an extender unit. The  
35 biosynthetic pathway for AF and ST is, approximately, as follows: a hexanoate starter  
unit is converted into an initial polyketide precursor (octaketide) which is converted into  
norsolorinic acid, which is converted into averantin, which is converted into averufanin,  
40 25 which is converted into averufin, which is converted into versiconal hemiacetal acetate,  
which is converted into versicolorin B, which is converted into versicolorin A, which is  
converted into demethylsterigmatocystin, which is converted into ST, which is converted  
45 into O-methylsterigmatocystin, which is converted into AFB<sub>1</sub>. See, Yu (1995) Journal of  
Bacteriology 177(16):4792-4800 and the references cited therein, and Silva (1996) JBC  
30 271(23):13600-13608 and the references cited therein.

5 The chemical structures of the important mycotoxin polyketides Aflatoxin  
B<sub>1</sub>, and compounds in the biosynthetic pathway for Aflatoxin B<sub>1</sub>, including  
sterigmatocystins, norisolomnic acid, and a variety of other compounds can be found,  
10 e.g., in Silva et al. (1996) JBC 271:23:13600-13608 and the references cited therein.

5 The double bond at carbon atoms 15,16 of AFB<sub>1</sub> is very important with  
respect to toxicity (Silva et al., 1996, *id*). This double bond is also susceptible to  
oxidations such as hydroxylation, epoxidation etc. These are monooxygenase catalyzed  
15 reactions. A number of monooxygenases, including P450s (*see* Ortiz de Montellano  
(ed.) 1995, Cytochrome P450 Structure and Mechanism and Biochemistry, Second  
10 Edition Plenum Press (New York and London), monooxygenase from *P. oleovorans* (J.  
Biol. Chem., 248, 1725-1730, 1973; May L. Am. Chem. Soc., 98, 7856-7858) and other  
20 homologous non-heme iron-sulfur monooxygenases from *Rhodococcus*, *Mycobacterium*,  
*ocardia*, *Pseudomonas* and *Bacillus*; heme-dependent peroxidases, iron-sulfur  
monooxygenases and quinone-dependent monooxygenases are known and can be  
25 recombined in the methods herein to provide mycotoxin resistant nucleic acids. Many of  
15 these detoxification enzymes confer increased hydrophilicity to the mycotoxin, thereby  
facilitating excretion, e.g., in mammals.

30 P450s are particularly preferred monooxygenases herein. P450s are a  
superfamily of enzymes capable of catalyzing a wide variety of reactions including  
20 epoxidation, hydroxylation, O-dealkylations, desaturation etc. As discussed herein, one  
way of eliminating the toxicity of AF and ST, trichothecenes (T) and fumonisins (F) is to  
35 shuffle and select for a monooxygenase such as P450 which is capable of oxidation of  
mycotoxins. In one preferred embodiment, this monooxygenase nucleic acid is  
transduced into crop plants to make the plants mycotoxin resistant. With respect to AF,  
40 25 oxidation at the 15,16 position provides detoxification. Oxidative changes in other  
positions (for example, O-demethylation of the methoxy group in position 8, *see*, Silva et  
al. 1996) also help render the molecule nontoxic.

45 One particularly preferred source of p450 nucleic acids for shuffling is the  
cyp 1, 2 and 3 families of genes, e.g., from humans. *See*,  
30 <http://drnelson.utmem.edu/homepage.html>. A feature of the invention is the discovery

5 that these genes display mycotoxin detoxification activity, making them especially suitable targets for recombination to develop improved detoxification properties.

10 While much of the discussion below deals explicitly with P450 monooxygenases, this is largely for clarity of illustration. The discussion is representative  
5 of the recombination strategies and chemistries and improvements which can be made to the structurally and functionally similar peroxidases and chlorperoxidases, as well as to the structurally unrelated iron-sulfur methane monooxygenases, trichothecene-3-O-  
15 acetyltransferase, 3-O-Methyltransferase, glutathione S-transferase, epoxide hydrolases, isomerases, macrolide-O-acetyltransferases, 3-O-acetyltransferases, and cis-diol  
10 producing monooxygenases for furan, as well as for non-monooxygenase genes which can catalyze detoxification reactions such as epoxidations, hydroxylations, O-  
20 dealkylations, desaturations, etc.

Gene shuffling and family shuffling provide two of the most powerful  
25 methods available for improving and "migrating" (gradually changing the type of reaction, substrate or activity of a selected enzyme) the functions of biocatalysts. In  
15 family shuffling, homologous sequences, e.g., from different species or chromosomal positions, are recombined. In gene shuffling, a single sequence is mutated or otherwise  
30 altered and then recombined.

The generation and screening of high quality shuffled libraries provides for  
20 DNA shuffling (or "directed evolution"). The availability of appropriate high-throughput analytical chemistry to screen the libraries permits integrated high-throughput shuffling  
35 and screening of the libraries to achieve a desired mycotoxin detoxification activity.

The invention provides significant advantages over previously used  
40 methods for optimization of mycotoxin detoxification genes. For example, DNA  
25 shuffling can result in optimization of a desirable property even in the absence of a detailed understanding of the mechanism by which the particular property is mediated.  
In addition, entirely new properties can be obtained upon shuffling of DNAs, i.e.,  
45 shuffled DNAs can encode polypeptides or RNAs with properties entirely absent in the parental DNAs which are shuffled. Indeed, even non-functional DNA sequences such as  
30 pseudo genes can be shuffled, particularly with homologous functional genes, to achieve new substrate specificity and activity.

5                   Sequence recombination can be achieved in many different formats and permutations of formats, as described in further detail below. These formats share some common principles.

10                   The targets for modification, vary in different applications, as does the  
5                   property sought to be acquired or improved. Examples of candidate targets for acquisition of a property or improvement in a property include genes that encode proteins which have enzymatic or other activities useful in monooxygenase or other detoxification  
15                   reactions.

                  The methods use at least two variant forms of a starting target. The  
10                   variant forms of candidate substrates can show substantial sequence or secondary structural similarity with each other, but they should also differ in at least one and preferably at least two positions. The initial diversity between forms can be the result of natural variation, *e.g.*, the different variant forms (homologs) are obtained from different  
20                   individuals or strains of an organism, or constitute related sequences from the same organism (*e.g.*, allelic variations), or constitute homologs from different organisms (interspecific variants). Alternatively, initial diversity can be induced, *e.g.*, the variant forms can be generated by error-prone transcription, such as an error-prone PCR or use  
25                   of a polymerase which lacks proof-reading activity (*see*, Liao (1990) *Gene* 88:107-111), of the first variant form, or, by replication of the first form in a mutator strain (mutator  
30                   host cells are discussed in further detail below, and are generally well known). The initial diversity between substrates is greatly augmented in subsequent steps of  
35                   recombination for library generation.

                  A mutator strain can include any mutants in any organism impaired in the functions of mismatch repair. These include mutant gene products of *mutS*, *mutT*,  
40                   25                   *mutH*, *mutL*, *ovrD*, *dcm*, *vsr*, *umuC*, *umuD*, *sbcB*, *recJ*, etc. The impairment is achieved by genetic mutation, allelic replacement, selective inhibition by an added reagent such as a small compound or an expressed antisense RNA, or other techniques. Impairment can be of the genes noted, or of homologous genes in any organism.

45                   The properties or characteristics that can be acquired or improved vary  
30                   widely, and, of course depend on the choice of substrate. For example, for monooxygenase genes, properties that one can improve include, but are not limited to,

5 increased range of monooxygenases activity encoded by a particular detoxification gene,  
increased potency against a mycotoxin target, increased expression level of the  
detoxification gene, increased tolerance of the protein encoded by the detoxification gene  
10 to protease degradation (or other natural protein or RNA degradative processes),  
5 increased detoxification activity ranges for conditions such as heat, cold, low or high pH,  
and reduced toxicity to the host cell.

15 At least two variant forms of a nucleic acid which can confer mycotoxin  
detoxification activity are recombined to produce a library of recombinant  
monooxygenase genes. The library is then screened to identify at least one recombinant  
10 gene that is optimized for the particular property or properties of interest.

20 Often, improvements are achieved after one round of recombination and  
selection. However, recursive sequence recombination can also be employed to achieve  
still further improvements in a desired property, or to bring about new (or "distinct")  
properties. Recursive sequence recombination entails successive cycles of recombination  
25 to generate molecular diversity. That is, one creates a family of nucleic acid molecules  
showing some sequence identity to each other but differing in the presence of mutations.  
In any given cycle, recombination can occur *in vivo* or *in vitro*, intracellularly or  
30 extracellularly. Furthermore, diversity resulting from recombination can be augmented in  
any cycle by applying prior methods of mutagenesis (*e.g.*, error-prone PCR or cassette  
20 mutagenesis) to either the substrates or products for recombination.

35 A recombination cycle is optionally followed by at least one cycle of  
screening or selection for molecules having a desired property or characteristic.  
Recombination cycles can also be repeated prior to selection to increase the diversity of a  
set of recombinant nucleic acids prior to selection. If a recombination cycle is performed  
40 *in vitro*, the products of recombination, *i.e.*, recombinant segments, are sometimes  
introduced into cells before the screening step. Recombinant segments can also be linked  
to an appropriate vector or other regulatory sequences before screening. Alternatively,  
45 products of recombination generated *in vitro* are sometimes packaged in viruses (*e.g.*,  
bacteriophage) before screening. If recombination is performed *in vivo*, recombination  
30 products can sometimes be screened in the cells in which recombination occurred. In

5 other applications, recombinant segments are extracted from the cells, and optionally packaged as viruses, before screening.

10 The nature of screening or selection depends on what property or characteristic is to be acquired or the property or characteristic for which improvement is  
5 sought, and many examples are discussed below. It is not usually necessary to understand the molecular basis by which particular products of recombination (recombinant segments) have acquired new or improved properties or characteristics relative to the  
15 starting substrates. For example, a mycotoxin detoxification gene can have many component sequences each having a different intended role (*e.g.*, coding sequence, regulatory sequences, targeting sequences, stability-conferring sequences, subunit  
10 sequences and sequences affecting integration). Each of these component sequences can be varied and recombined simultaneously. Screening/selection can then be performed, for example, for recombinant segments that have increased ability to confer mycotoxin  
20 detoxification activity upon a cell without the need to attribute such improvement to any of the individual component sequences of the vector.

25 Depending on the particular screening protocol used for a desired property, initial round(s) of screening can sometimes be performed using bacterial cells due to high transfection efficiencies and ease of culture. However, especially for  
30 eukaryotic mycotoxin detoxification enzymes such as eukaryotic P450s, yeast, fungal or other eukaryotic systems are optionally used for library expression and screening.  
20 Similarly other types of screening which are not amenable to screening in bacterial or simple eukaryotic library cells, are performed in cells selected for use in an environment  
35 close to that of their intended use. Final rounds of screening can be performed in the precise cell type of intended use.

40 25 If further improvement in a property is desired, at least one, and usually a collection, of recombinant segments surviving a first round of screening/selection are subject to a further round of recombination. These recombinant segments can be  
45 recombined with each other or with exogenous segments representing the original substrates or further variants thereof. Again, recombination can proceed *in vitro* or *in vivo*. If the previous screening step identifies desired recombinant segments as  
30 components of cells, the components can be subjected to further recombination *in vivo*,



5 or can be subjected to further recombination *in vitro*, or can be isolated before  
performing a round of *in vitro* recombination. Conversely, if the previous screening step  
identifies desired recombinant segments in naked form or as components of viruses, these  
10 segments can be introduced into cells to perform a round of *in vivo* recombination. The  
5 second round of recombination, irrespective how performed, generates further  
recombinant segments which encompass additional diversity than is present in  
15 recombinant segments resulting from previous rounds.

The second round of recombination can be followed by a further round of  
screening/selection according to the principles discussed above for the first round. The  
10 stringency of screening/selection can be increased between rounds. Also, the nature of  
20 the screen and the property being screened for can vary between rounds if improvement  
in more than one property is desired or if acquiring more than one new property is  
desired. Additional rounds of recombination and screening can then be performed until  
25 the recombinant segments have sufficiently evolved to acquire the desired new or  
15 improved property or function.

The practice of this invention involves the construction of recombinant  
nucleic acids and the expression of genes in transfected host cells. Molecular cloning  
30 techniques to achieve these ends are known in the art. A wide variety of cloning and *in*  
*vitro* amplification methods suitable for the construction of recombinant nucleic acids  
20 such as expression vectors are well-known to persons of skill. General texts which  
describe molecular biological techniques useful herein, including mutagenesis, include  
35 Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology  
volume 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook et al., Molecular  
Cloning - A Laboratory Manual (2nd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory,  
40 25 Cold Spring Harbor, New York, 1989 ("Sambrook") and Current Protocols in Molecular  
Biology, F.M. Ausubel et al., eds., Current Protocols, a joint venture between Greene  
Publishing Associates, Inc. and John Wiley & Sons, Inc., (supplemented through 1998)  
45 ("Ausubel"). Examples of techniques sufficient to direct persons of skill through *in*  
*vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase  
30 chain reaction (LCR), Q $\beta$ -replicase amplification and other RNA polymerase mediated  
techniques (e.g., NASBA) are found in Berger, Sambrook, and Ausubel, as well as

5 Mullis *et al.*, (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis *et al.* eds) Academic Press Inc. San Diego, CA (1990) (Innis);  
10 Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research*  
5 (1991) 3, 81-94; (Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86, 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 1874; Lomell *et al.* (1989) *J. Clin. Chem* 35, 1826; Landegren *et al.*, (1988) *Science* 241, 1077-1080; Van Brunt (1990) *Biotechnology* 8, 291-294; Wu and Wallace, (1989) *Gene* 4, 560; Barringer *et al.* (1990) *Gene* 89, 117,  
15 and Sooknanan and Malek (1995) *Biotechnology* 13: 563-564. Improved methods of cloning *in vitro* amplified nucleic acids are described in Wallace *et al.*, U.S. Pat. No. 5,426,039. Improved methods of amplifying large nucleic acids by PCR are summarized  
10 in Cheng *et al.* (1994) *Nature* 369: 684-685 and the references therein, in which PCR amplicons of up to 40kb are generated. One of skill will appreciate that essentially any RNA can be converted into a double stranded DNA suitable for restriction digestion, PCR expansion and sequencing using reverse transcriptase and a polymerase. See,  
20 Ausbel, Sambrook and Berger, *all supra*.

Oligonucleotides for use as probes, *e.g.*, in *in vitro* amplification methods, for use as gene probes, or as shuffling targets (*e.g.*, synthetic genes or gene segments) are typically synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981), *Tetrahedron Letts.*,  
30 22(20):1859-1862, *e.g.*, using an automated synthesizer, as described in Needham-VanDevanter *et al.* (1984) *Nucleic Acids Res.*, 12:6159-6168.  
20 Oligonucleotides can also be custom made and ordered from a variety of commercial sources known to persons of skill.

Indeed, essentially any nucleic acid with a known sequence can be custom  
25 ordered from any of a variety of commercial sources, such as The Midland Certified Reagent Company (mcrc@oligos.com), The Great American Gene Company (<http://www.genco.com>), ExpressGen Inc. ([www.expressgen.com](http://www.expressgen.com)), Operon Technologies Inc. (Alameda, CA) and many others. Similarly, peptides and antibodies  
40 can be custom ordered from any of a variety of sources, such as PeptidoGenic (pkim@ccnet.com), HTI Bio-products, inc. (<http://www.htibio.com>), BMA Biomedicals Ltd (U.K.), Bio-Synthesis, Inc., and many others.  
30

### Family Shuffling Mycotoxin Detoxification genes

A large number of P450 genes are known. This makes P450 genes ideal substrates for family shuffling (any of the other genes for mycotoxin detoxification which are discussed supra are For identification of homologous genes used in family shuffling strategies, representative alignments of P450 enzymes can be found in the Appendices of the volume Cytochrome P450: Structure, Mechanism, and Biochemistry, 2<sup>nd</sup> Addition (ed. By Paul R. Ortiz de Montellano) Plenum Press, New York, 1995) ("Ortiz de Montellano"). An up-to-date list of P450s can be found electronically on the World Wide Web (<http://www.drnelson.utm.edu/homepage.html>).

To illustrate the family shuffling approach to improving P450 enzymes (essentially similar approaches apply to other mycotoxin detoxification nucleic acids), one or more of the more than 1000 members of this superfamily is/are selected, aligned with similar homologous sequences, and shuffled against these homologous sequences.

An example P450 which can be shuffled by any of the procedures herein is the *Aspergillus nidulans stcL* gene, which encodes a cytochrome P450 monooxygenase required for bisfuran desaturation during aflatoxin/sterigmatocystin biosynthesis. See, Kelkar et al (1997) JBC 1589-1594 for a description of the structure and function of the *stcL* gene.

Similarly, gene sequences for other monooxygenases, trichothecene-3-O-acetyltransferase, a 3-O-Methyltransferase, a glutathione S-transferase, epoxide hydrolases, isomerases, macrolide-O-acetyltransferases, 3-O-acetyltransferases, and cis-diol producing monooxygenases specific for furan are all well illustrated in the literature and in, e.g., publicly available sequence repositories such as Genbank.

For example, Trichothecene mycotoxins such as deoxynivalenol, 4,15-diacetoxyscripenol and T-2 toxin are all potent protein synthesis inhibitors for eukaryotic organisms. The 3-O-acetyl derivatives of these toxins have significantly lower toxic activity. *Tri101*, a gene responsible for 3-O-acetylation reactions was cloned from a *Fusarium graminearum* cDNA library. See, Kimura et al. (1997) JBC 273(3):1654-1661 for a description of the gene sequence. Kimura et al. cloned the *Tri101* cDNA for expression in yeast and selected for 3-O-acetylation in the presence of T-2 toxin. See, *id.* The *Tri101* gene can be shuffled by any of the procedures herein. The gene specifying

5 this enzyme codes a 451-amino acid protein, which is unique (Kimura et al. 1998). This  
gene can be shuffled for improvement of activity as well as broadening specificity with  
respect to acetylation of hydroxyl groups in other mycotoxins like ST and F. The  
10 selection system for T and other mycotoxins that are protein synthesis inhibitors is  
straight forward. Yeast is susceptible to these toxins and any transformed yeast capable  
of acetylating the toxins will be viable (Kimura et al. 1998).

15 Other sources of genes for shuffling include those catalyzing N-  
acetylation, O-glycosylation and O-phosphorylation. These are mechanisms of  
inactivation of representatives of various antibiotics and herbicides. Genes specifying  
10 these activities can be shuffled both for improvement of activity and specificity with  
respect to compounds like T and ST and F. The gene or genes optimized for any of the  
above transferase activity can be cloned into desired crops in order to detoxify one or  
20 more pathogen-derived mycotoxins.

25 Other genes for detoxification of mycotoxins include the 3-O-  
Methyltransferases (MT). These enzymes provide for irreversible modification to ether.  
MTs are typically single polypeptides with no redox cofactors. Selection in yeast is used  
to evolve and screen as that used for cloning of 3-OAT specific for mycotoxins of the  
30 family T. T2 (or related 3-deacetyl derivative) is used with radioactive (Methyl) labeled  
SAM to screen for source organisms capable of modifying T2.

20 Another candidate for DNA-shuffling to inactivate various mycotoxins is  
the gene coding for macrohalide-O-acyltransferase such as 3-O-acyltransferase (Hara and  
Hutchinson (1992) J. Bacteriol. 174: 5141-5144. This gene is shuffled individually or in  
35 combination with its homologs for the desired activity with mycotoxins.

40 Targets for shuffling to acquire mycotoxin detoxification properties also  
25 include mycotoxin synthetic genes such as polyketide synthases. These synthetic genes  
could be modified by shuffling to catalyze reverse synthetic reactions to break down the  
polyketides they ordinarily produce. An example target is the *pksST* gene from  
*Aspergillus nidulans*, which is necessary for the synthesis of ST. Yu and Leonard (1995)  
45 Journal of Bacteriology 177(16):4792-4800 describe the structure and function of the  
*pksST* gene. Similarly, the *pksL* gene required for aflatoxin biosynthesis in *Aspergillus*  
30 *parasiticus* is described by Feng and Leonard Journal of Bacteriology 177(21):6246-

5 6254. Another example is Versicolorin B synthase, which synthesizes the side chain  
cyclization of racemic versiconal hemiacetal to the bisfuran ring system of versicolorin B.  
The dihydrobisfuran is important to the mutagenic nature of AFB<sub>1</sub> and ST. The isolation  
10 and characterization of the Versicolorin B synthase gene from *Aspergillus parasiticus* and  
5 the partial characterization of the related synthetic cluster is described in Silva (1996)  
JBC 271(23):13600-13608. A total of twenty five co-regulated transcripts defining a ST  
gene cluster, and containing most, or all, of the genes necessary for ST biosynthesis in *A.*  
15 *nidulans* is described by Brown et al. (1996) PNAS 93:1418-1422. These genes are also  
targets for shuffling for mycotoxin detoxification. Whole genome shuffling approaches  
10 (described below) can also be used to select for plant cells which produce products that  
20 down-regulate production of genes such as those described in Brown, *id.* (which provide  
for ST and AF biosynthesis) thereby reducing ST and AF levels in target plants.

A variety of organisms known to contain additional monooxygenases  
which could be shuffled in the methods of the invention are also known. The most  
25 15 comprehensive studies on bacterial alkene epoxidation have been done on *Pseudomonas*  
*oleovorans*. Work on *P. oleovorans* by May and coworkers (J. Biol. Chem., 248, 1725-  
1730) showed that the monooxygenase contained in the cells is capable of epoxidizing  
30 octene to 1,2-epoxy-octane in 70% enantiomeric purity. In addition, this enzyme is  
capable of converting 1,7-octadiene to the diepoxide (May et al, J. Am. Chem. Soc., 98,  
20 7856-7858) and 1,5-hexadiene and 1,11-dodecadiene to epoxides. This enzyme system is  
also capable of mediating hydroxylation of longer chain alkanes (octanes, etc.) and fatty  
35 acids. The enzyme has been cloned and sequenced and is comprised of three protein  
components: rubredoxin (mw 19,000), NADH-rubredoxin reductase, and the  $\omega$ -  
hydroxylase (a non-heme iron protein). Microorganisms having MMO enzyme activities  
40 25 with similar properties include the genera *Rhodococcus*, *Mycobacterium*, *Nocardia*  
(*Nocardia carollina* B-276) and *Pseudomonas Corynebacterium equi* (IFO 3730). All of  
these strains are available from ATCC and serve as sources for the genes which can be  
isolated by hybridization and gene amplification methods.

45 30 Mycotoxin detoxification screening is done most easily in yeast, but a  
bacterial system could also be constructed by co-expressing the accessory electron  
transport proteins adrenodoxin and adrenodoxin reductase. DNA from clones with

improved activity can be shuffled together in subsequent rounds of DNA shuffling and screened for further improvement.

Assays for Mycotoxin Inactivation

Screening a number of cloned cytochrome P450 monooxygenases for activity vs. various AF and ST and other mycotoxins yields P450 nucleic acids specifying these reactions and other oxidative changes in 15, or 16 positions (like hydroxylation). These P450 genes can be of fungal, microbial, plant, insect or mammalian origin. The screen can be conducted by measuring the activity against any particular mycotoxin or against multiple mycotoxins, e.g., by preparing extracts of clones expressing P450 genes. The expected product(s) from the above toxins as well as other oxidized products derived from a P450 can be identified based on differences in physical properties (oxidation of mycotoxins causes a detectable difference in the physical characteristics of mycotoxins).

It is possible to directly select the clones expressing P450 specifying either specific or broad-based oxidation by using yeast, if the yeast are susceptible to the compound. For example, as noted above, Kimura et al (1997) describe expression of the *trl101* gene in yeast and selection of the yeast in medium containing T-2 toxin, a potent mycotoxin. This same assay format can be used for any mycotoxin which is toxic to yeast, or inhibitory to yeast growth on a medium.

Similarly, such assays can be performed using any of a variety of other cultured cells, by growing the cells (e.g., prokaryotic or eukaryotic cells) in the presence of a mycotoxin. To gradually select for more and more potent mycotoxin detoxification nucleic acids, cells are grown in medium containing increasingly high doses of the mycotoxin, e.g., following each round of a reiterative DNA shuffling procedure, as described herein.

In general, the culture of cells, including yeast, animal cells, plant cells and the like are well known. In addition to Berger, Ausubel and Sambrook, *all supra*, details on animal cell culture can be found in Freshney (*Culture of Animal Cells, a Manual of Basic Technique, third edition* Wiley-Liss, New York (1994)) and the references cited therein. The culture of plant cells is described, e.g., in Payne et al. (1992) Plant Cell and Tissue Culture in Liquid Systems John Wiley & Sons, Inc. New York, NY (Payne); and Gamborg and Phillips (eds) (1995) Plant Cell, Tissue and Organ

5 Culture: Fundamental Methods Springer Lab Manual, Springer-Verlag (Berlin  
Heidelberg New York) (Gamborg). A variety of Cell culture media are described in  
10 Atlas and Parks (eds) The Handbook of Microbiological Media (1993) CRC Press, Boca  
Raton, FL (Atlas). It will be appreciated that plant cells are desirably transduced with  
5 mycotoxin resistance nucleic acids to reduce food contamination by mycotoxins and to  
improve plant resistance to mycotoxins, e.g., to enhance yield. Accordingly, it can be  
15 convenient to screen to mycotoxin detoxification using plant cells in culture which  
correspond to plants desirably transduced.

If the oxidized products of the reaction are fluorescent, clones having  
10 mycotoxin detoxification activity are detected by fluorescence of specific mycotoxins.  
The intensity of fluorescence may help select clones having higher activity (or higher  
20 expression).

Clones expressing shuffled mycotoxin detoxification nucleic acids can be  
25 examined for oxidation of one or more mycotoxin in pools of 10, in order to prescreen  
15 the initial transformants rapidly. Any pools showing significant activity can be  
deconvoluted to identify single desirable clones with high activity and/or broad  
specificity. The mycotoxin detoxification nucleic acid from one or more such clones  
30 could be subjected to a second and subsequent round of shuffling in order to further  
optimize the rate of oxidation or to broaden the mycotoxin substrate specificity.

20 The appropriate gene or genes optimized for rapid oxidation of one or  
more mycotoxins like AF, ST, T and F are optionally cloned into desired crops in order  
35 rapidly detoxify the toxin produced by the pathogen. This reduces food contamination  
caused by these compounds. In the case of AF and ST, even if the oxidized product  
were the toxic 15,16-epoxide or an equivalent derivative of the parent substrate, it would  
40 25 be sequestered quickly in the plants due to its instability. For example, the epoxide could  
be rapidly conjugated to a nucleophile or hydrolyzed or it could form a DNA-adduct. All  
of these derivatives, if present in the grain commodities produced from transgenic plants,  
45 are nontoxic to humans and animals.

For detection of demethylation (other than MS), free thiol or amine -  
30 bearing scintillating polymeric beads (covalent reaction with epoxy moiety) can be used.  
The beads are washed, radioactivity counted (only beads attached to O-methylated T2



will be counted). In a variation of assay, with non-bead scintillating material, surfaces are activated with thiols or amines. All these variations are, in essence, SPA assays.

For Glutathione S-transferases (GSTs), the epoxide moiety of T2 is amenable to nucleophilic attack by thiol nucleophiles, including glutathione, whether transferred or not by GST. The thiol-T2 conjugate compound can be formed in an irreversible manner and is not an active toxin. Endogenous GST levels in plants are likely to be sufficient. Selection in yeast is used to evolve and screen as above for 3-OAT. DNA shuffling is used to optimize the specificity of plant GST enzymes towards T2 epoxide.

For epoxide hydrolase or isomerase it is sufficient to disrupt the T2 toxophore by modifying 12,13-epoxide to a glycol or an aldehyde. No known natural enzymes work on this epoxide. However, DNA shuffling is used to impart and optimize the required specificity. Selection in yeast is used to evolve and screen as for 3-OAT above.

Other assays for shuffling include chemical assays based on reactivity of residual epoxide, or formed rearranged 13-aldehyde product. One option is the use of a cytochrome P450 enzyme for aflatoxin detoxification by epoxidation of the double bond of the dihydrobisfuran moiety. Also, this can be used in conjunction with nucleophilic opening of the 15,16-epoxide (epoxide hydrolase, or GST, or an amine nucleophile, e.g. nucleobase or amino acid). Although 13-acetal can, in principle, be a subject to hydrolytic opening (enzymatic), the spontaneous toxophore regeneration may occur as it is favored by stereochemical means. Reactivity of 15,16-epoxide towards nucleophiles can be used for screen of P450s with the best activity towards AFB1. Exogenously supplied nucleophiles convenient for detection of AFB-epoxide-nucleophile adduct can be supplied in order to have shuffling done in bacterial or other microbial host which is insensitive to AFB1.

An alternative method for the assay of P450s with optimized activity towards aflatoxinB1 can use a variation of scintillation proximity assay using beads or other SPA material activated with a suitable nucleophilic group (amine, thiol) to trap any AFB1 15,16-epoxide. This uses a radioactively labeled AFB1 as a screen substrate. The latter can be prepared by chemical means, or by means of biosynthesis (with AFB1



5 producing *Aspergillus* strains) using a radioactively labeled AFB1 precursor/intermediate of its biosynthesis pathway.

10 Other microbial genes specifying oxygenases suitable for oxidation of aflatoxin such as AFB1 are cis-diol making dioxygenases with specificity for furan substructures. Other genes coding for flavoprotein monooxygenases of microbial or 5 mammalian origin are suitable candidates for shuffling for generating and improving activity vs. AF, ST and T. An way of degrading AFB1 is by opening the coumarin-lactone ring - C1 ester with a hydrolase. This is of value when the ring opening product is intercepted with a peroxidase for oxidative radical coupling of the free C12 phenol. 15 The toxophore is destroyed after this oxidation. Even without peroxidase-induced radical oxidation process, the hydrolytic option is of use because the lactone ring opening product is subject to spontaneous C-1 hydrolytic decarboxylation (beta-ketoacid). 20

As is apparent from the foregoing, the relevant assay will depend on the application. Many assays formats are suitable. Advantageously, any of the assays can be 25 practiced in a high-throughput format. 15

In the high throughput assays of the invention, it is possible to screen up to several thousand different shuffled variants in a single day. For example, each well of a 30 microtiter plate can be used to run a separate assay, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single variant. Thus, a single standard microtiter plate can assay about 100 (e.g., 96) mycotoxin detoxification 20 reactions. If 1536 well plates are used, then a single plate can easily assay from about 100- about 1500 different reactions. It is possible to assay several different plates per 35 day; assay screens for up to about 6,000-20,000 different assays (i.e., involving different nucleic acids, encoded proteins, concentrations, etc.) is possible using the integrated systems of the invention. More recently, microfluidic approaches to reagent 40 manipulation have been developed, e.g., by Caliper Technologies (Palo Alto, CA). 25

In addition to fluidic approaches, it is possible simply to grow cells on plates of agar which comprise mycotoxins. Cells which have mycotoxin detoxification 45 activity (e.g., due to transduction with mycotoxin detoxification nucleic acids) are able to grow on the plates. This approach offers a very simple and high-throughput screening 30 method. 50

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In one aspect, library members, e.g., cells, viral plaques, spores or the like, are separated on solid media to produce individual colonies (or plaques). Using an automated colony picker (e.g., the Q-bot, Genetix, U.K.), colonies or plaques are identified, picked, and up to 10,000 different mutants inoculated into 96 well microtitre dishes, optionally containing glass balls in the wells to prevent aggregation. The Q-bot does not pick an entire colony but rather inserts a pin through the center of the colony and exits with a small sampling of cells, (or mycelia) and spores (or viruses in plaque applications). The time the pin is in the colony, the number of dips to inoculate the culture medium, and the time the pin is in that medium each effect inoculum size, and each can be controlled and optimized. The uniform process of the Q-bot decreases human handling error and increases the rate of establishing cultures (roughly 10,000/4 hours). These cultures are then shaken in a temperature and humidity controlled incubator. The glass balls in the microtiter plates act to promote uniform aeration of cells dispersal of mycelial fragments, or the like, similar to the blades of a fermenter. Clones from cultures of interest can be cloned by limiting dilution. As also described supra, plaques or cells constituting libraries can also be screened directly for production of proteins, either by detecting hybridization, protein activity, protein binding to antibodies, or the like.

The ability to detect a subtle increase in the performance of a shuffled library member over that of a parent strain relies on the sensitivity of the assay. The chance of finding the organisms having an improvement in mycotoxin detoxification activity is increased by the number of individual mutants that can be screened by the assay. To increase the chances of identifying a pool of sufficient size, a prescreen that increases the number of mutants processed by 10-fold can be used. The goal of the primary screen will be to quickly identify mutants having equal or better product titres than the parent strain(s) and to move only these mutants forward to liquid cell culture for subsequent analysis.

A number of well known robotic systems have also been developed for solution phase chemistries useful in assay systems. These systems include automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms

5 (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, Hewlett-Packard, Palo Alto, Calif.) which mimic the manual synthetic operations performed by a scientist. Any of  
10 the above devices are suitable for use with the present invention, e.g., for high-throughput screening of molecules encoded by codon-altered nucleic acids. The nature and implementation of modifications to these devices (if any) so that they can operate as  
15 discussed herein with reference to the integrated system will be apparent to persons skilled in the relevant art.

High throughput screening systems are commercially available (*see, e.g.,* Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman  
10 Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, *etc.*). These systems typically automate entire procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid  
25 start up as well as a high degree of flexibility and customization.

15 The manufacturers of such systems provide detailed protocols the various high throughput. Thus, for example, Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand  
30 binding, and the like. Microfluidic approaches to reagent manipulation have also been developed, e.g., by Caliper Technologies (Palo Alto, CA).

20 Optical images viewed (and, optionally, recorded) by a camera or other recording device (*e.g.,* a photodiode and data storage device) are optionally further  
35 processed in any of the embodiments herein, *e.g.,* by digitizing the image and/or storing and analyzing the image on a computer. As noted above, in some applications, mycotoxin detoxification products are fluorescent, making optical detection approaches  
40 appropriate in these instances. A variety of commercially available peripheral equipment and software is available for digitizing, storing and analyzing a digitized video or digitized optical image, *e.g.,* using PC (Intel x86 or pentium chip- compatible DOS™, OS2™ WINDOWS™, WINDOWS NT™ or WINDOWS95™ based machines),  
45 MACINTOSH™, or UNIX based (*e.g.,* SUN™ work station) computers.

30 One conventional system carries light from the assay device to a cooled charge-coupled device (CCD) camera, in common use in the art. A CCD camera  
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5 includes an array of picture elements (pixels). The light from the specimen is imaged on  
the CCD. Particular pixels corresponding to regions of the specimen (e.g., individual  
10 hybridization sites on an array of biological polymers) are sampled to obtain light  
intensity readings for each position. Multiple pixels are processed in parallel to increase  
5 speed. The apparatus and methods of the invention are easily used for viewing any  
sample, e.g., by fluorescent or dark field microscopic techniques.

15 Integrated systems for analysis in the present invention typically include a  
digital computer with high-throughput liquid control software, image analysis software,  
data interpretation software, a robotic liquid control armature for transferring solutions  
10 from a source to a destination operably linked to the digital computer, an input device  
(e.g., a computer keyboard) for entering data to the digital computer to control high  
20 throughput liquid transfer by the robotic liquid control armature and, optionally, an  
image scanner for digitizing label signals from labeled assay component. The image  
scanner interfaces with the image analysis software to provide a measurement of optical  
25 intensity. Typically, the intensity measurement is interpreted by the data interpretation  
15 software to show whether the mycotoxin detoxification products are produced.

30 Monooxygenase activity can also be monitored by HPLC, gas  
chromatography and mass spectroscopy, as well as a variety of other analytical methods  
available to one of skill. Incorporation of  $^{18}\text{O}$  from radio-labeled molecular oxygen can  
20 be monitored directly by mass shift by MS methods and by an appropriate radioisotope  
detector with HPLC and GC devices. In a high-throughput modality, a method of choice  
35 is high-throughput MS, or MS with an electron spray-based detection method.

40 In addition, epoxide formation can be indirectly measured by various  
reactive colorimetric reactions. For example, disappearance of peroxide over time can  
25 be monitored directly either potentiometrically or colorimetrically using a number of  
commercially available peroxide reactive dyes.

45 In one set of assays, the relative toxicity of mycotoxin products produced  
by modification of mycotoxin detoxification enzymes is determined. In particular,  
toxicity can be evaluated in any of the usual assays for mycotoxin toxicity and,  
30 optionally, compared to the toxicity of the unmodified mycotoxin. In the event that  
toxicity is reduced, secondary toxic effects of detoxification products can be evaluated

5 using these usual assays for mycotoxin activity, or using additional assays such as cell  
survival assays, e.g., in the presence of increasing levels of the secondary product. This  
secondary assay helps to determine which mycotoxin detoxification activities are most  
10 desirable, i.e., using secondary toxicities of mycotoxin metabolites as a measure of  
unwanted toxicity.

#### Formats for Nucleic Acid Recombination-Nucleic Acid Shuffling

15 The present invention involves shuffling of nucleic acids. The following  
publications describe a variety of recursive recombination (shuffling) procedures and/or  
methods which can be incorporated into such procedures: Stemmer, et al., (1999)  
10 "Molecular breeding of viruses for targeting and other clinical properties. Tumor  
Targeting" 4:1-4; Nasset al. (1999) "DNA Shuffling of subgenomic sequences of  
subtilisin" Nature Biotechnology 17:893-896; Chang et al. (1999) "Evolution of a cytokine  
using DNA family shuffling" Nature Biotechnology 17:793-797; Minshull and Stemmer  
25 (1999) "Protein evolution by molecular breeding" Current Opinion in Chemical Biology  
3:284-290; Christians et al. (1999) "Directed evolution of thymidine kinase for AZT  
phosphorylation using DNA family shuffling" Nature Biotechnology 17:259-264;  
Crameriet al. (1998) "DNA shuffling of a family of genes from diverse species accelerates  
30 directed evolution" Nature 391:288-291; Crameri et al. (1997) "Molecular evolution of an  
arsenate detoxification pathway by DNA shuffling," Nature Biotechnology 15:436-438;  
20 Zhang et al. (1997) "Directed evolution of an effective fucosidase from a galactosidase by  
DNA shuffling and screening" Proceedings of the National Academy of Sciences, U.S.A.  
35 94:4504-4509; Patten et al. (1997) "Applications of DNA Shuffling to Pharmaceuticals and  
Vaccines" Current Opinion in Biotechnology 8:724-733; Crameri et al. (1996)  
"Construction and evolution of antibody-phage libraries by DNA shuffling" Nature  
40 25 Medicine 2:100-103; Crameri et al. (1996) "Improved green fluorescent protein by  
molecular evolution using DNA shuffling" Nature Biotechnology 14:315-319; Gates et al.  
(1996) "Affinity selective isolation of ligands from peptide libraries through display on a  
lac repressor 'headpiece dimer'" Journal of Molecular Biology 255:373-386; Stemmer  
45 (1996) "Sexual PCR and Assembly PCR" In: The Encyclopedia of Molecular Biology.  
VCH Publishers, New York. pp.447-457; Crameri and Stemmer (1995) "Combinatorial  
30 multiple cassette mutagenesis creates all the permutations of mutant and wildtype  
cassettes" BioTechniques 18:194-195; Stemmer et al., (1995) "Single-step assembly of a

5 gene and entire plasmid form large numbers of oligodeoxyribonucleotides" Gene, 164:49-53; Stemmer (1995) "The Evolution of Molecular Computation" Science 270: 1510; Stemmer (1995) "Searching Sequence Space" Bio/Technology 13:549-553; Stemmer  
10 (1994) "Rapid evolution of a protein in vitro by DNA shuffling" Nature 370:389-391; and Stemmer (1994) "DNA shuffling by random fragmentation and reassembly: In vitro  
5 recombination for molecular evolution." Proceedings of the National Academy of Sciences, U.S.A. 91:10747-10751.

15 Additional details regarding DNA shuffling methods are found in U.S. Patents by the inventors and their co-workers, including: United States Patent 5,605,793 to Stemmer (February 25, 1997), "METHODS FOR IN VITRO RECOMBINATION;"  
10 United States Patent 5,811,238 to Stemmer et al. (September 22, 1998) "METHODS FOR GENERATING POLYNUCLEOTIDES HAVING DESIRED CHARACTERISTICS BY  
20 ITERATIVE SELECTION AND RECOMBINATION;" United States Patent 5,830,721 to Stemmer et al. (November 3, 1998), "DNA MUTAGENESIS BY RANDOM  
25 FRAGMENTATION AND REASSEMBLY;" United States Patent 5,834,252 to Stemmer, et al. (November 10, 1998) "END-COMPLEMENTARY POLYMERASE REACTION,"  
15 and United States Patent 5,837,458 to Minshull, et al. (November 17, 1998), "METHODS AND COMPOSITIONS FOR CELLULAR AND METABOLIC ENGINEERING."

30 In addition, details and formats for DNA shuffling are found in a variety of PCT and foreign patent application publications, including: Stemmer and Crameri, "DNA  
20 MUTAGENESIS BY RANDOM FRAGMENTATION AND REASSEMBLY" WO 95/22625; Stemmer and Lipschutz "END COMPLEMENTARY POLYMERASE CHAIN  
35 REACTION" WO 96/33207; Stemmer and Crameri "METHODS FOR GENERATING POLYNUCLEOTIDES HAVING DESIRED CHARACTERISTICS BY ITERATIVE  
25 SELECTION AND RECOMBINATION" WO 97/0078; Minshull and Stemmer, "METHODS AND COMPOSITIONS FOR CELLULAR AND METABOLIC  
40 ENGINEERING" WO 97/35966; Punnonen et al. "TARGETING OF GENETIC VACCINE VECTORS" WO 99/41402; Punnonen et al. "ANTIGEN LIBRARY  
45 IMMUNIZATION" WO 99/41383; Punnonen et al. "GENETIC VACCINE VECTOR ENGINEERING" WO 99/41369; Punnonen et al. OPTIMIZATION OF  
30 IMMUNOMODULATORY PROPERTIES OF GENETIC VACCINES WO 99/41368; Stemmer and Crameri, "DNA MUTAGENESIS BY RANDOM FRAGMENTATION  
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5 AND REASSEMBLY" EP 0934999; Stemmer "EVOLVING CELLULAR DNA UPTAKE  
BY RECURSIVE SEQUENCE RECOMBINATION" EP 0932670; Stemmer et al.,  
10 "MODIFICATION OF VIRUS TROPISM AND HOST RANGE BY VIRAL GENOME  
SHUFFLING" WO 9923107; Apt et al., "HUMAN PAPILLOMAVIRUS VECTORS" WO  
5 9921979; Del Cardayre et al. "EVOLUTION OF WHOLE CELLS AND ORGANISMS  
BY RECURSIVE SEQUENCE RECOMBINATION" WO 9831837; Patten and Stemmer,  
15 "METHODS AND COMPOSITIONS FOR POLYPEPTIDE ENGINEERING" WO  
9827230; Stemmer et al., and "METHODS FOR OPTIMIZATION OF GENE THERAPY  
BY RECURSIVE SEQUENCE SHUFFLING AND SELECTION" WO9813487.

10 Certain U.S. Applications provide additional details regarding DNA  
shuffling and related techniques, including "SHUFFLING OF CODON ALTERED  
20 GENES" by Patten et al. filed September 29, 1998, (USSN 60/102,362), January 29, 1999  
(USSN 60/117,729), and September 28, 1999, USSN \_\_\_\_\_ (Attorney Docket Number 20-  
28520US/PCT); "EVOLUTION OF WHOLE CELLS AND ORGANISMS BY  
25 RECURSIVE SEQUENCE RECOMBINATION", by del Cardyre et al. filed July 15, 1998  
(USSN 09/166,188), and July 15, 1999 (USSN 09/354,922); "OLIGONUCLEOTIDE  
15 MEDIATED NUCLEIC ACID RECOMBINATION" by Crameri et al., filed February 5,  
1999 (USSN 60/118,813) and filed June 24, 1999 (USSN 60/141,049) and filed September  
30 28, 1999 (USSN \_\_\_\_\_, Attorney Docket Number 02-29620US); and "USE OF CODON-  
20 BASED OLIGONUCLEOTIDE SYNTHESIS FOR SYNTHETIC SHUFFLING" by  
Welch et al., filed September 28, 1999 (USSN \_\_\_\_\_, Attorney Docket Number 02-  
35 010070US); and "METHODS FOR MAKING CHARACTER STRINGS,  
POLYNUCLEOTIDES & POLYPEPTIDES HAVING DESIRED CHARACTERISTICS"  
by Selifonov and Stemmer, filed February 5, 1999 (USSN 60/118854).

40 25 As review of the foregoing publications, patents, published applications and  
U.S. patent applications reveals, shuffling (or "recursive recombination") of nucleic acids  
to provide new nucleic acids with desired properties can be carried out by a number of  
45 methods. These methods can be adapted to the present invention to evolve the mycotoxin  
detoxification activity as discussed herein to produce new mycotoxin detoxification nucleic  
30 acids with new or improved properties. Both the methods of making such mycotoxin  
detoxification nucleic acids and the mycotoxin detoxification nucleic acids produced by  
these methods are a feature of the invention.



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In brief, at least 5 different general classes of recombination methods are applicable to the present invention. First, nucleic acids can be recombined in vitro by any of a variety of techniques discussed in the references above, including e.g., DNase digestion of nucleic acids to be recombined followed by ligation and/or PCR reassembly of the nucleic acids. Second, nucleic acids can be recursively recombined in vivo, e.g., by allowing recombination to occur between nucleic acids in cells. Third, whole cell genome recombination methods can be used in which whole genomes of cells are recombined, optionally including spiking of the genomic recombination mixtures with desired library components such as mycotoxin detoxification nucleic acids homologue nucleic acids. Fourth, synthetic recombination methods can be used, in which oligonucleotides corresponding to different mycotoxin detoxification nucleic acid homologues are synthesized and reassembled in PCR or ligation reactions which include oligonucleotides which correspond to more than one parental nucleic acid, thereby generating new recombined nucleic acids. Oligonucleotides can be made by standard nucleotide addition methods, or can be made and shuffled by tri-nucleotide synthetic and shuffling approaches. Fifth, in silico methods of recombination can be effected in which genetic algorithms are used in a computer to recombine sequence strings which correspond to mycotoxin detoxification nucleic acid homologues. The resulting recombined sequence strings are optionally converted into nucleic acids by synthesis of nucleic acids which correspond to the recombined sequences, e.g., in concert with oligonucleotide synthesis/ gene reassembly techniques. Any of the preceding general recombination formats can be practiced in a reiterative fashion to generate a more diverse set of recombinant nucleic acids. In addition, these general approaches can, and often are, used in combination.

The above references provide these and other basic recombination formats as well as many modifications of these formats. Regardless of the format which is used, the nucleic acids of the invention can be recombined (with each other or with related (or even unrelated) nucleic acids to produce a diverse set of recombinant nucleic acids, including homologous nucleic acids. In general, the sequence recombination techniques described herein provide particular advantages in that they provide for recombination between mycotoxin detoxification nucleic acids, or derivatives thereof, in any available format, thereby providing a very fast way of exploring the manner in which different combinations of sequences can affect a desired result.

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Following recombination, any nucleic acids which are produced can be  
selected for a desired activity. In the context of the present invention, this can include  
testing for and identifying any mycotoxin detoxification activities, by any of the assays in  
10 the art. In addition, useful properties such as low crop yield enhancement, can also be  
simultaneously selected for. A variety of mycotoxin detoxification nucleic acid related (or  
even unrelated) properties can be assayed for, using any available assay.

15 A recombinant nucleic acid produced by recursively recombining one or  
more polynucleotide of the invention with one or more additional nucleic acid also forms a  
part of the invention. The one or more additional nucleic acid may include another  
10 polynucleotide of the invention (i.e., one or more evolved mycotoxin detoxification nucleic  
acids); optionally, alternatively, or in addition, the one or more additional nucleic acid can  
include, e.g., a nucleic acid encoding a naturally-occurring mycotoxin detoxification  
nucleic acid or a subsequence thereof, or any homologous sequence or subsequence  
thereof, or, e.g., any other homologous or non-homologous nucleic acid (certain  
25 recombination formats noted above, notably those performed synthetically or in silico, do  
not require homology for recombination).

The recombining steps may be performed in vivo, in vitro, or in silico as  
described in more detail in the references above. Also included in the invention is a cell  
30 containing any resulting recombinant nucleic acid, nucleic acid libraries produced by  
recursive recombination of the nucleic acids set forth herein, and populations of cells,  
vectors, viruses, plasmids or the like comprising the library or comprising any recombinant  
nucleic acid resulting from recombination (or recursive recombination) of a nucleic acid as  
set forth herein with another such nucleic acid, or an additional nucleic acid.  
Corresponding sequence strings in a database present in a computer system or computer  
25 readable medium are a feature of the invention.

#### 40 Specific Formats for Sequence Recombination

DNA shuffling can be applied to a collection of mycotoxin without prior  
screening for activity vs. one or more mycotoxins. The shuffled genes can be cloned in  
45 appropriate *E. coli* or yeast, and clones exhibiting desired activity can be selected as  
described above. The screening will be based e.g., on differences in the physical  
30 properties between the parent mycotoxin and its modified, oxidized product, or upon cell  
survival on mycotoxin containing media. The final gene product can be optimized for

5 rapid oxidation and/or desired substrate specificity for one or more mycotoxins, by  
further rounds of shuffling. The optimized gene or genes obtained after several rounds  
10 of shuffling could be cloned into desired crops in which AF, ST or other mycotoxin  
production by the appropriate pathogen is a problem. This will help eliminate the  
5 toxicity associated with the pathogen-produced mycotoxins in the grains.

Shuffled gene(s) developed by the above screening method for the  
15 identification of oxidation of one or more mycotoxins are optionally shuffled by at least  
one of the five general approaches for sequence recombination noted above.

In specific formats, DNA-shuffling can be performed on a single gene.

10 Alternatively, several homologous genes can be identified by sequence comparison with  
known homologous genes. These genes can be synthesized and shuffled as a family of  
homologs, to select recombinants with the desired activity. The shuffled genes can be  
25 cloned into *E. coli*, yeast, plants, fungi, or animal cells and those producing high activity  
can be identified by the methods described above.

15 Whole genome shuffling can be performed to shuffle detoxification genes  
(along with other genomic nucleic acids), thereby producing cells with enhanced  
detoxification activity. For whole genome shuffling approaches, it is not even necessary  
30 to identify which mycotoxin detoxification genes are being shuffled. Instead, e.g., plant  
cell genomes are combined and shuffled to acquire mycotoxin detoxification activity, as  
20 measured in any of the assays above.

35 Mycotoxin detoxification genes can be codon modified to access  
mutational diversity not present in any naturally occurring detoxification gene. Shuffling  
can be performed using synthetic shuffling and in silico approaches. Details on each of  
40 these procedures can be found in the references noted above and as further detailed  
25 below.

Generally, the methods of the invention entail performing DNA  
45 recombination ("shuffling") and screening or selection to "evolve" individual genes,  
whole plasmids or viruses, multigene clusters, or even whole genomes (e.g., Stemmer  
(1995) *Bio/Technology* 13:549-553 and the other references noted herein). Reiterative  
30 cycles of recombination and screening/selection can be performed to further evolve the  
nucleic acids of interest. Such techniques do not require the extensive analysis and  
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5 computation required by conventional methods for polypeptide engineering. Shuffling  
allows the recombination of large numbers of mutations in a minimum number of  
10 selection cycles, in contrast to natural pair-wise recombination events (e.g., as occur  
during sexual replication). Thus, the sequence recombination techniques described  
5 herein provide particular advantages in that they provide recombination between  
mutations in any or all of these, thereby providing a very fast way of exploring the  
15 manner in which different combinations of mutations can affect a desired result. In some  
instances, however, structural and/or functional information is available which, although  
not required for sequence recombination, provides opportunities for modification of the  
10 technique.

20 Exemplary formats and examples for sequence recombination, referred to,  
e.g., as "DNA shuffling," "fast forced evolution," or "molecular breeding," have been  
described by the present inventors and co-workers in the publications, patents and patent  
25 applications noted above.

15 In one class of embodiments, the recombination procedure starts with at  
least two substrates that generally show substantial sequence identity to each other (*i.e.*,  
at least about 30%, 50%, 70%, 80% or 90% sequence identity), but differ from each  
30 other at certain positions. The difference can be any type of mutation, for example,  
substitutions, insertions and deletions. Often, different segments differ from each other  
20 in about 5-20 positions. For recombination to generate increased diversity relative to the  
starting materials, the starting materials must differ from each other in at least two  
35 nucleotide positions. That is, if there are only two substrates, there should be at least  
two divergent positions. If there are three substrates, for example, one substrate can  
40 differ from the second at a single position, and the second can differ from the third at a  
25 different single position. The starting DNA segments can be natural variants of each  
other, for example, allelic or species variants. The segments can also be from nonallelic  
45 genes showing some degree of structural and usually functional relatedness (*e.g.*,  
different genes within a superfamily, such as the cytochrome P450 super family). The  
starting DNA segments can also be induced variants of each other. For example, one  
30 DNA segment can be produced by error-prone PCR replication of the other, or by  
substitution of a mutagenic cassette. Induced mutants can also be prepared by

5 propagating one (or both) of the segments in a mutagenic strain. In these situations,  
strictly speaking, the second DNA segment is not a single segment but a large family of  
10 related segments. The different segments forming the starting materials are often the  
same length or substantially the same length. However, this need not be the case; for  
5 example; one segment can be a subsequence of another. The segments can be present as  
part of larger molecules, such as vectors, or can be in isolated form.

15 The starting DNA segments are recombined by any of the sequence  
recombination formats provided herein to generate a diverse library of recombinant DNA  
segments. Such a library can vary widely in size from having fewer than 10 to more than  
10  $10^5$ ,  $10^9$ ,  $10^{12}$  or more members. In some embodiments, the starting segments and the  
20 recombinant libraries generated will include full-length coding sequences and any  
essential regulatory sequences, such as a promoter and polyadenylation sequence,  
required for expression. In other embodiments, the recombinant DNA segments in the  
25 library can be inserted into a common vector providing sequences necessary for  
15 expression before performing screening/selection.

#### *Use of Restriction Enzyme Sites to Recombine Mutations*

30 In some situations it is advantageous to use restriction enzyme sites in  
nucleic acids to direct the recombination of mutations in a nucleic acid sequence of  
interest. These techniques are particularly preferred in the evolution of fragments that  
20 cannot readily be shuffled by existing methods due to the presence of repeated DNA or  
other problematic primary sequence motifs. These situations also include recombination  
35 formats in which it is preferred to retain certain sequences unmutated. The use of  
restriction enzyme sites is also preferred for shuffling large fragments (typically greater  
than 10 kb), such as gene clusters that cannot be readily shuffled and "PCR-amplified"  
40 because of their size. Although fragments up to 50 kb have been reported to be amplified  
25 by PCR (Barnes, *Proc. Natl. Acad. Sci. U.S.A.* 91:2216-2220 (1994)), it can be  
problematic for fragments over 10 kb, and thus alternative methods for shuffling in the  
45 range of 10 - 50 kb and beyond are preferred. Preferably, the restriction endonucleases  
used are of the Class II type (Sambrook, Ausubel and Berger, *supra*) and of these,  
30 preferably those which generate nonpalindromic sticky end overhangs such as AlwI, Sfi  
I or BstXI. These enzymes generate nonpalindromic ends that allow for efficient ordered

5 reassembly with DNA ligase. Typically, restriction enzyme (or endonuclease) sites are  
identified by conventional restriction enzyme mapping techniques (Sambrook, Ausubel,  
10 and Berger, *supra.*), by analysis of sequence information for that gene, or by  
introduction of desired restriction sites into a nucleic acid sequence by synthesis (*i.e.* by  
5 incorporation of silent mutations).

The DNA substrate molecules to be digested can either be from *in vivo*  
15 replicated DNA, such as a plasmid preparation, or from PCR amplified nucleic acid  
fragments harboring the restriction enzyme recognition sites of interest, preferably near  
the ends of the fragment. Typically, at least two variants of a gene of interest, each  
10 having one or more mutations, are digested with at least one restriction enzyme  
determined to cut within the nucleic acid sequence of interest. The restriction fragments  
20 are then joined with DNA ligase to generate full length genes having shuffled regions.  
The number of regions shuffled will depend on the number of cuts within the nucleic acid  
sequence of interest. The shuffled molecules can be introduced into cells as described  
25 above and screened or selected for a desired property as described herein. Nucleic acid  
15 can then be isolated from pools (libraries), or clones having desired properties and  
subjected to the same procedure until a desired degree of improvement is obtained.

30 In some embodiments, at least one DNA substrate molecule or fragment  
thereof is isolated and subjected to mutagenesis. In some embodiments, the pool or  
20 library of religated restriction fragments are subjected to mutagenesis before the  
digestion-ligation process is repeated. "Mutagenesis" as used herein comprises such  
35 techniques known in the art as PCR mutagenesis, oligonucleotide-directed mutagenesis,  
site-directed mutagenesis, etc., and recursive sequence recombination by any of the  
techniques described herein.

#### 40 25 *Reassembly PCR*

A further technique for recombining mutations in a nucleic acid sequence  
utilizes "reassembly PCR." This method can be used to assemble multiple segments that  
45 have been separately evolved into a full length nucleic acid template such as a gene. This  
technique is performed when a pool of advantageous mutants is known from previous  
30 work or has been identified by screening mutants that may have been created by any  
mutagenesis technique known in the art, such as PCR mutagenesis, cassette mutagenesis,  
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5 doped oligo mutagenesis, chemical mutagenesis, or propagation of the DNA template *in*  
10 *vivo* in mutator strains. Boundaries defining segments of a nucleic acid sequence of  
interest preferably lie in intergenic regions, introns, or areas of a gene not likely to have  
5 mutations of interest. Preferably, oligonucleotide primers (oligos) are synthesized for  
PCR amplification of segments of the nucleic acid sequence of interest, such that the  
15 sequences of the oligonucleotides overlap the junctions of two segments. The overlap  
region is typically about 10 to 100 nucleotides in length. Each of the segments is  
amplified with a set of such primers. The PCR products are then "reassembled"  
20 according to assembly protocols such as those discussed herein to assemble randomly  
fragmented genes. In brief, in an assembly protocol the PCR products are first purified  
away from the primers, hy, for example, gel electrophoresis or size exclusion  
25 chromatography. Purified products are mixed together and subjected to about 1-10 cycles  
of denaturing, reannealing, and extension in the presence of polymerase and  
deoxynucleoside triphosphates (dNTP's) and appropriate buffer salts in the absence of  
15 additional primers ("self-priming"). Subsequent PCR with primers flanking the gene are  
used to amplify the yield of the fully reassembled and shuffled genes.

30 In some embodiments, the resulting reassembled genes are subjected to  
mutagenesis before the process is repeated.

35 In a further embodiment, the PCR primers for amplification of segments  
of the nucleic acid sequence of interest are used to introduce variation into the gene of  
interest as follows. Mutations at sites of interest in a nucleic acid sequence are identified  
by screening or selection, by sequencing homologues of the nucleic acid sequence, and so  
40 on. Oligonucleotide PCR primers are then synthesized which encode wild type or mutant  
information at sites of interest. These primers are then used in PCR mutagenesis to  
25 generate libraries of full length genes encoding permutations of wild type and mutant  
information at the designated positions. This technique is typically advantageous in cases  
where the screening or selection process is expensive, cumbersome, or impractical  
45 relative to the cost of sequencing the genes of mutants of interest and synthesizing  
mutagenic oligonucleotides.

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Oligonucleotide and in silico shuffling formats for MycotoxinDetoxification Shuffling

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Two additional related formats are useful in the practice of the present invention. The first, referred to as "in silico" shuffling utilizes computer algorithms to perform "virtual" shuffling using genetic operators in a computer. As applied to the present invention, mycotoxin detoxification nucleic acid sequence strings are recombined in a computer system and desirable products are made, e.g., by reassembly PCR or ligation of synthetic oligonucleotides, or other available techniques. In silico shuffling is described in detail in Selifonov and Stemmer in "METHODS FOR MAKING CHARACTER STRINGS, POLYNUCLEOTIDES & POLYPEPTIDES HAVING DESIRED CHARACTERISTICS" filed 02/05/1999, USSN 60/118854. In brief, genetic operators (algorithms which represent given genetic events such as point mutations, recombination of two strands of homologous nucleic acids, etc.) are used to model recombinational or mutational events which can occur in one or more nucleic acid, e.g., by aligning nucleic acid sequence strings (using standard alignment software, or by manual inspection and alignment) and predicting recombinational outcomes based upon selected genetic algorithms (mutation, recombination, etc.). The predicted recombinational outcomes are used to produce corresponding molecules, e.g., by oligonucleotide synthesis and reassembly PCR. As applied to the present invention, mycotoxin detoxification nucleic acids are aligned and recombined in silico, using any desired genetic operator, to produce mycotoxin detoxification character strings which are then generated synthetically for subsequent screening.

The second useful format is referred to as "oligonucleotide mediated shuffling" in which oligonucleotides corresponding to a family of related homologous nucleic acids (e.g., as applied to the present invention, families of homologous mycotoxin detoxification variants of a nucleic acid) which are recombined to produce selectable nucleic acids. This format is described in detail in Crameri et al. "OLIGONUCLEOTIDE MEDIATED NUCLEIC ACID RECOMBINATION" filed February 5, 1999, USSN 60/118,813 and Crameri et al. "OLIGONUCLEOTIDE MEDIATED NUCLEIC ACID RECOMBINATION" filed June 24, 1999, USSN 60/141,049. In brief, selected oligonucleotides corresponding to multiple homologous parental nucleic acids are



5 synthesized, ligated and elongated (typically in a recursive format), typically either in a  
polymerase or ligase-mediated elongation reaction, to produce full-length mycotoxin  
detoxification nucleic acids. The technique can be used to recombine homologous or even  
10 non-homologous mycotoxin detoxification nucleic acid sequences.

5 One advantage of oligonucleotide-mediated recombination is the ability to  
recombine homologous nucleic acids with low sequence similarity, or even non-  
homologous nucleic acids. In these low-homology oligonucleotide shuffling methods, one  
15 or more set of fragmented nucleic acids (e.g., oligonucleotides corresponding to multiple  
mycotoxin detoxification nucleic acids) are recombined, e.g., with a set of crossover  
family diversity oligonucleotides. Each of these crossover oligonucleotides have a  
20 plurality of sequence diversity domains corresponding to a plurality of sequence diversity  
domains from homologous or non-homologous nucleic acids with low sequence similarity.  
The fragmented oligonucleotides, which are derived by comparison to one or more  
homologous or non-homologous nucleic acids, can hybridize to one or more region of the  
25 crossover oligos, facilitating recombination.  
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When recombining homologous nucleic acids, sets of overlapping family  
gene shuffling oligonucleotides (which are derived by comparison of homologous nucleic  
30 acids, by synthesis of corresponding oligonucleotides) are hybridized and elongated (e.g.,  
by reassembly PCR or ligation), providing a population of recombined nucleic acids,  
20 which can be selected for a desired trait or property. The set of overlapping family  
shuffling gene oligonucleotides includes a plurality of oligonucleotide member types  
35 which have consensus region subsequences derived from a plurality of homologous target  
nucleic acids.

Typically, as applied to the present invention, family gene shuffling  
40 25 oligonucleotide which include one or more mycotoxin detoxification nucleic acid(s) are  
provided by aligning homologous nucleic acid sequences to select conserved regions of  
sequence identity and regions of sequence diversity. A plurality of family gene shuffling  
oligonucleotides are synthesized (serially or in parallel) which correspond to at least one  
45 region of sequence diversity.

30 Sets of fragments, or subsets of fragments used in oligonucleotide shuffling  
approaches can be provided by cleaving one or more homologous nucleic acids (e.g., with  
a DNase), or, more commonly, by synthesizing a set of oligonucleotides corresponding to a  
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5 plurality of regions of at least one nucleic acid (typically oligonucleotides corresponding to  
a full-length nucleic acid are provided as members of a set of nucleic acid fragments). In  
the shuffling procedures herein, these cleavage fragments can be used in conjunction with  
10 family gene shuffling oligonucleotides, e.g., in one or more recombination reaction to  
5 produce recombinant mycotoxin detoxification nucleic acid(s).

One final synthetic variant worth noting is found in "SHUFFLING OF  
CODON ALTERED GENES" by Patten et al. filed September 29, 1998, (USSN  
15 60/102,362), January 29, 1999 (USSN 60/117,729), and September 28, 1999,  
USSN \_\_\_\_\_ (Attorney Docket Number 20-28520US/PCT). As noted in detail in this set  
10 of related applications, one way of generating diversity in a set of nucleic acids to be  
shuffled (i.e., as applied to the present invention, mycotoxin detoxification nucleic acids),  
20 is to provide codon-altered nucleic acids which can be shuffled to provide access to  
sequence space not present in naturally occurring sequences. In brief, by synthesizing  
nucleic acids in which the codons which encode polypeptides are altered, it is possible to  
25 access a completely different mutational spectrum upon subsequent mutation of the  
nucleic acid. This increases the sequence diversity of the starting nucleic acids for  
shuffling protocols, which alters the rate and results of forced evolution procedures.  
Codon modification procedures can be used to modify any mycotoxin detoxification  
30 nucleic acid herein, e.g., prior to performing DNA shuffling.

20 In brief, oligonucleotide sets comprising codon variations are synthesized  
and reassembled into full-length nucleic acids. The oligonucleotide sets can themselves be  
35 shuffled (e.g., where the oligonucleotides to be reassembled provide sequence diversity at  
selected sites), and/or the full-length sequences can be shuffled by any available procedure  
to produce diverse sets of mycotoxin detoxification nucleic acids.

25 *Site Directed Mutagenesis (SDM) with Oligonucleotides Encoding Homologue  
Mutations Followed by Shuffling*

In some embodiments of the invention, sequence information from one or  
more substrate sequences is added to a given "parental" sequence of interest, with  
45 subsequent recombination between rounds of screening or selection. Typically, this is  
done with site-directed mutagenesis performed by techniques well known in the art (e.g.,  
30 Berger, Ausubel and Sambrook, *supra.*), or by the oligonucleotide or in silico methods

5 noted above, with one substrate as template and oligonucleotides encoding single or  
multiple mutations from other substrate sequences, e.g. homologous genes. After  
10 screening or selection for an improved phenotype of interest, the selected recombinant(s)  
can be further evolved using RSR techniques described herein. After screening or  
5 selection, site-directed mutagenesis can be done again with another collection of  
oligonucleotides encoding homologue mutations, and the above process repeated until the  
15 desired properties are obtained.

When the difference between two homologues is one or more single point  
mutations in a codon, degenerate oligonucleotides can be used that encode the sequences  
10 in both homologues. One oligonucleotide can include many such degenerate codons and  
still allow one to exhaustively search all permutations over that block of sequence.  
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When the homologue sequence space is very large, it can be advantageous  
to restrict the search to certain variants. Thus, for example, computer modeling tools  
25 (Lathrop *et al.* (1996) *J. Mol. Biol.*, 255: 641-665) can be used to model each homologue  
15 mutation onto the target protein and discard any mutations that are predicted to grossly  
disrupt structure and function.

#### *In Vitro DNA Shuffling Formats*

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In one embodiment for shuffling DNA sequences *in vitro*, the initial  
substrates for recombination are a pool of related sequences, e.g., different variant  
20 forms, as homologs from different individuals, strains, or species of an organism, or  
35 related sequences from the same organism, as allelic variations. The sequences can be  
DNA or RNA and can be of various lengths depending on the size of the gene or DNA  
fragment to be recombined or reassembled. Preferably the sequences are from 40 base  
40 pairs (bp) to 50 kilobases (kb).

25 The pool of related substrates are converted into overlapping fragments,  
e.g., from about 5 bp to 5 kb or more. Often, for example, the size of the fragments is  
from about 10 bp to 1000 bp, e.g., about 30 or 40 bp to about 100bp, e.g., about 100 bp  
45 to 500 bp. The conversion can be effected by a number of different methods, such as  
DNase I or RNase digestion, random shearing, partial restriction enzyme digestion or  
30 oligonucleotide synthesis. For discussions of protocols for the isolation, manipulation,  
enzymatic digestion, and the like of nucleic acids, see, for example, Sambrook *et al.* and  
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Ausubel, both *supra*. The concentration of nucleic acid fragments of a particular length and sequence is often less than 0.1 % or 1% by weight of the total nucleic acid. The number of different specific nucleic acid fragments in the mixture is usually at least about 100, 500 or 1000.

The mixed population of nucleic acid fragments are converted to at least partially single-stranded form using a variety of techniques, including, for example, heating, chemical denaturation, use of DNA binding proteins, and the like. Conversion can be effected by heating to about 80°C to 100°C, more preferably from 90°C to 96°C, to form single-stranded nucleic acid fragments and then reannealing. Conversion can also be effected by treatment with single-stranded DNA binding protein (see Wold (1997) *Annu. Rev. Biochem.* 66:61-92) or *recA* protein (see, e.g., Kiiianitsa (1997) *Proc. Natl. Acad. Sci. U S A* 94:7837-7840). Single-stranded nucleic acid fragments having regions of sequence identity with other single-stranded nucleic acid fragments can then be reannealed by cooling to 20°C to 75°C, and preferably from 40°C to 65°C.

Renaturation can be accelerated by the addition of polyethylene glycol (PEG), other volume-excluding reagents or salt. The salt concentration is preferably from 0 mM to 200 mM, more preferably the salt concentration is from 10 mM to 100 mM. The salt may be KCl or NaCl. The concentration of PEG is preferably from 0% to 20%, more preferably from 5% to 10%. The fragments that reanneal can be from different substrates. The annealed nucleic acid fragments are incubated in the presence of a nucleic acid polymerase, such as Taq or Klenow, and dNTP's (*i.e.* dATP, dCTP, dGTP and dTTP). If regions of sequence identity are large, Taq polymerase can be used with an annealing temperature of between 45-65°C. If the areas of identity are small, Klenow polymerase can be used with an annealing temperature of between 20-30°C. The polymerase can be added to the random nucleic acid fragments prior to annealing, simultaneously with annealing or after annealing.

The process of denaturation, renaturation and incubation in the presence of polymerase of overlapping fragments to generate a collection of polynucleotides containing different permutations of fragments is sometimes referred to as shuffling of the nucleic acid *in vitro*. This cycle is optionally repeated for a desired number of times.

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Preferably the cycle is repeated from 2 to 100 times, more preferably the sequence is repeated from 10 to 40 times. The resulting nucleic acids are a selectable family of double-stranded polynucleotides of from about 50 bp to about 100 kb, preferably from 500 bp to 50 kb. The population represents variants of the starting substrates showing substantial sequence identity thereto but also diverging at several positions. The population has many more members than the starting substrates. The population of fragments resulting from shuffling is used to transform host cells, optionally after cloning into a vector.

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In one embodiment utilizing *in vitro* shuffling, subsequences of recombination substrates can be generated by amplifying the full-length sequences under conditions which produce a substantial fraction, typically at least 20 percent or more, of incompletely extended amplification products. Another embodiment uses random primers to prime the entire template DNA to generate less than full length amplification products. The amplification products, including the incompletely extended amplification products are denatured and subjected to at least one additional cycle of reannealing and amplification. This variation, in which at least one cycle of reannealing and amplification provides a substantial fraction of incompletely extended products, is termed "stuttering." In the subsequent amplification round, the partially extended (less than full length) products reanneal to and prime extension on different sequence-related template species. In another embodiment, the conversion of substrates to fragments can be effected by partial PCR amplification of substrates.

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In another embodiment, a mixture of fragments is spiked with one or more oligonucleotides. The oligonucleotides can be designed to include precharacterized mutations of a wildtype sequence, or sites of natural variations between individuals or species. The oligonucleotides also include sufficient sequence or structural homology flanking such mutations or variations to allow annealing with the wildtype fragments. Annealing temperatures can be adjusted depending on the length of homology.

In a further embodiment, recombination occurs in at least one cycle by template switching, such as when a DNA fragment derived from one template primes on the homologous position of a related but different template. Template switching can be induced by addition of recA (*see*, Kiianitsa (1997) *supra*), rad51 (*see*, Namsaraev (1997)

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5 *Mol. Cell. Biol.* 17:5359-5368), rad55 (see, Clever (1997) *EMBO J.* 16:2535-2544),  
rad57 (see, Sung (1997) *Genes Dev.* 11:1111-1121) or other polymerases (e.g., viral  
10 polymerases, reverse transcriptase) to the amplification mixture. Template switching can  
also be increased by increasing the DNA template concentration.

5 Another embodiment utilizes at least one cycle of amplification, which can  
be conducted using a collection of overlapping single-stranded DNA fragments of related  
15 sequence, and different lengths. Fragments can be prepared using a single stranded DNA  
phage, such as M13 (see, Wang (1997) *Biochemistry* 36:9486-9492). Each fragment can  
hybridize to and prime polynucleotide chain extension of a second fragment from the  
10 collection, thus forming sequence-recombined polynucleotides. In a further variation,  
20 ssDNA fragments of variable length can be generated from a single primer by Pfu, Taq,  
Vent, Deep Vent, UITma DNA polymerase or other DNA polymerases on a first DNA  
template (see, Cline (1996) *Nucleic Acids Res.* 24:3546-3551). The single stranded DNA  
25 fragments are used as primers for a second, Kunkel-type template, consisting of a  
15 uracil-containing circular ssDNA. This results in multiple substitutions of the first  
template into the second. See, Levichkin (1995) *Mol. Biology* 29:572-577; Jung (1992)  
*Gene* 121:17-24.

30 In some embodiments of the invention, shuffled nucleic acids obtained by  
use of the recursive recombination methods of the invention, are put into a cell and/or  
20 organism for screening. Shuffled monooxygenase genes can be introduced into, for  
example, bacterial cells, yeast cells, fungal cells vertebrate cells, invertebrate cells or  
35 plant cells for initial screening. *Bacillus* species (such as *B. subtilis* and *E. coli* are two  
examples of suitable bacterial cells into which one can insert and express shuffled  
monooxygenase genes which provide for convenient shuttling to other cell types (a  
40 25 variety of vectors for shuttling material between these bacterial cells and eukaryotic cells  
are available; see, Sambrook, Ausubel and Berger, *all supra*). The shuffled genes can be  
introduced into bacterial, fungal or yeast cells either by integration into the chromosomal  
45 DNA or as plasmids.

50 Although bacterial and yeast systems are most preferred in the present  
30 invention, in one embodiment, shuffled genes can also be introduced into plant cells for  
production purposes (it will be appreciated that transgenic plants are, increasingly, an

important source of industrial enzymes). Thus, a transgene of interest can be modified using the recursive sequence recombination methods of the invention *in vitro* and reinserted into the cell for *in vivo/in situ* selection for the new or improved monooxygenase property, in bacteria, eukaryotic cells, or whole eukaryotic organisms.

#### *In Vivo DNA Shuffling Formats*

In some embodiments of the invention, DNA substrate molecules are introduced into cells, wherein the cellular machinery directs their recombination. For example, a library of mutants is constructed and screened or selected for mutants with improved phenotypes by any of the techniques described herein. The DNA substrate molecules encoding the best candidates are recovered by any of the techniques described herein, then fragmented and used to transfect a plant host and screened or selected for improved function. If further improvement is desired, the DNA substrate molecules are recovered from the host cell, such as by PCR, and the process is repeated until a desired level of improvement is obtained. In some embodiments, the fragments are denatured and reannealed prior to transfection, coated with recombination stimulating proteins such as *recA*, or co-transfected with a selectable marker such as *Neo<sup>R</sup>* to allow the positive selection for cells receiving recombined versions of the gene of interest. Methods for *in vivo* shuffling are described in, for example, PCT application WO 98/13487 and WO 97/20078.

The efficiency of *in vivo* shuffling can be enhanced by increasing the copy number of a gene of interest in the host cells. For example, the majority of bacterial cells in stationary phase cultures grown in rich media contain two, four or eight genomes. In minimal medium the cells contain one or two genomes. The number of genomes per bacterial cell thus depends on the growth rate of the cell as it enters stationary phase. This is because rapidly growing cells contain multiple replication forks, resulting in several genomes in the cells after termination. The number of genomes is strain dependent, although all strains tested have more than one chromosome in stationary phase. The number of genomes in stationary phase cells decreases with time. This appears to be due to fragmentation and degradation of entire chromosomes, similar to apoptosis in mammalian cells. This fragmentation of genomes in cells containing multiple genome copies results in massive recombination and mutagenesis. The presence

5 of multiple genome copies in such cells results in a higher frequency of homologous  
recombination in these cells, both between copies of a gene in different genomes within  
10 the cell, and between a genome within the cell and a transfected fragment. The increased  
frequency of recombination allows one to evolve a gene evolved more quickly to acquire  
5 optimized characteristics.

15 In nature, the existence of multiple genomic copies in a cell type would  
usually not be advantageous due to the greater nutritional requirements needed to  
maintain this copy number. However, artificial conditions can be devised to select for  
high copy number. Modified cells having recombinant genomes are grown in rich media  
20 (in which conditions, multicopy number should not be a disadvantage) and exposed to a  
mutagen, such as ultraviolet or gamma irradiation or a chemical mutagen, *e.g.*,  
mitomycin, nitrous acid, photoactivated psoralens, alone or in combination, which  
induces DNA breaks amenable to repair by recombination. These conditions select for  
25 cells having multicopy number due to the greater efficiency with which mutations can be  
excised. Modified cells surviving exposure to mutagen are enriched for cells with  
15 multiple genome copies. If desired, selected cells can be individually analyzed for  
genome copy number (*e.g.*, by quantitative hybridization with appropriate controls). For  
30 example, individual cells can be sorted using a cell sorter for those cells containing more  
DNA, *e.g.*, using DNA specific fluorescent compounds or sorting for increased size  
20 using light dispersion. Some or all of the collection of cells surviving selection are tested  
35 for the presence of a gene that is optimized for the desired property.

In one embodiment, phage libraries are made and recombined in mutator  
strains such as cells with mutant or impaired gene products of *mutS*, *mutT*, *mutH*, *mutL*,  
40 *ovrD*, *dcm*, *vsr*, *umuC*, *umuD*, *sbcB*, *recJ*, etc. The impairment is achieved by genetic  
25 mutation, allelic replacement, selective inhibition by an added reagent such as a small  
compound or an expressed antisense RNA, or other techniques. High multiplicity of  
infection (MOI) libraries are used to infect the cells to increase recombination frequency.

45 Additional strategies for making phage libraries and or for recombining  
DNA from donor and recipient cells are set forth in U.S. Pat. No. 5,521,077.

30 Additional recombination strategies for recombining plasmids in yeast are set forth in  
WO 97 07205.

Recursive macroshuffling techniques are described in U.S. Pat. 5,811,238 to Stemmer.

#### *Whole Genome Shuffling*

In one embodiment, the selection methods herein are utilized in a "whole genome shuffling" format. An extensive guide to the many forms of whole genome shuffling is found in the pioneering application to the inventors and their co-workers entitled "Evolution of Whole Cells and Organisms by Recursive Sequence Recombination," by del Cardyre et al. e.g., WO98/31837, PCT/US99/15972, and 09/354,922.

In brief, whole genome shuffling makes no presuppositions at all regarding what nucleic acids may confer a desired property. Instead, entire genomes (e.g., from a genomic library, or isolated from an organism) are shuffled in cells and selection protocols applied to the cells. Thus, as applied to the present invention, cell genomes or sub genomes (e.g., libraries) are recombined and resulting recombinant cells comprising the recombined nucleic acids are selected for mycotoxin detoxification activity. Thus, one feature of the invention is a cell (e.g., plant, animal, bacterial, or even fungal cell which comprises mycotoxin detoxification activity). These cells can be used to produce anti-mycotoxin extracts or can be applied to reduce fungal growth and/or pathogenicity in a system of interest.

#### *Use of RecA*

The frequency of recombination between nucleic acids in the mycotoxin detoxification shuffling procedures herein can be increased by coating the nucleic acids with a recombinogenic protein, e.g., before or after introduction into cells. See Pati et al., *Molecular Biology of Cancer* 1, 1 (1996); Sena & Zarling, *Nature Genetics* 3, 365 (1996); Revet et al., *J. Mol. Biol.* 232, 779-791 (1993); Kowalczkowski & Zarling in *Gene Targeting* (CRC 1995), Ch. 7. The recombinogenic protein promotes homologous pairing and/or strand exchange. The best characterized *recA* protein is from *E. coli* and is available from Pharmacia (Piscataway, NJ). In addition to the wild-type protein, a number of mutant *recA*-like proteins have been identified (e.g., *recA803*). Further, many organisms have *recA*-like recombinases with strand-transfer activities (e.g., Ogawa et al., *Cold Spring Harbor Symposium on Quantitative Biology* 18, 567-576 (1993);



Johnson & Symington, *Mol. Cell. Biol.* 15, 4843-4850 (1995); Fugisawa et al., *Nucl. Acids Res.* 13, 7473 (1985); Hsieh et al., *Cell* 44, 885 (1986); Hsieh et al., *J. Biol. Chem.* 264, 5089 (1989); Fishel et al., *Proc. Natl. Acad. Sci. USA* 85, 3683 (1988); Cassuto et al., *Mol. Gen. Genet.* 208, 10 (1987); Ganea et al., *Mol. Cell Biol.* 7, 3124 (1987); Moore et al., *J. Biol. Chem.* 19, 11108 (1990); Keene et al., *Nucl. Acids Res.* 12, 3057 (1984); Kimiec, *Cold Spring Harbor Symp.* 48, 675 (1984); Kimeic, *Cell* 44, 545 (1986); Kolodner et al., *Proc. Natl. Acad. Sci. USA* 84, 5560 (1987); Sugino et al., *Proc. Natl. Acad. Sci. USA* 85, 3683 (1985); Halbrook et al., *J. Biol. Chem.* 264, 21403 (1989); Eisen et al., *Proc. Natl. Acad. Sci. USA* 85, 7481 (1988); McCarthy et al., *Proc. Natl. Acad. Sci. USA* 85, 5854 (1988); Lowenhaupt et al., *J. Biol. Chem.* 264, 20568 (1989). Examples of such recombinase proteins include *recA*, *recA803*, *uvrX*, (Roca, A.I., *Crit. Rev. Biochem. Molec. Biol.* 25, 415 (1990)), *sepl* (Kolodner et al., *Proc. Natl. Acad. Sci. (U.S.A.)* 84, 5560 (1987); Tishkoff et al., *Molec. Cell. Biol.* 11, 2593), *RuvC* (Dunderdale et al., *Nature* 354, 506 (1991)), *DST2*, *KEM1*, *XRN1* (Dykstra et al., *Molec. Cell. Biol.* 11, 2583 (1991)), *STPa/DST1* (Clark et al., *Molec. Cell. Biol.* 11, 2576 (1991)), *HPP-1* (Moore et al., *Proc. Natl. Acad. Sci. (U.S.A.)* 88, 9067 (1991)), other eukaryotic recombinases (Bishop et al., *Cell* 69, 439 (1992); Shinohara et al., *Cell* 69, 457. *RecA* protein forms a nucleoprotein filament when it coats a single-stranded DNA. In this nucleoprotein filament, one monomer of *recA* protein is bound to about 3 nucleotides. This property of *recA* to coat single-stranded DNA is essentially sequence independent, although particular sequences favor initial loading of *recA* onto a polynucleotide (e.g., nucleation sequences). The nucleoprotein filament(s) can be formed on essentially any DNA to be shuffled and can form complexes with both single-stranded and double-stranded DNA in procaryotic and eukaryotic cells. *RecA* mediated techniques are also found in WO/93/22443.

Before contacting with *recA* or other recombinase, mycotoxin detoxification fragments are optionally denatured, e.g., by heat-treatment. *RecA* protein is then added at a concentration of about 1-10  $\mu$ M. After incubation, the *recA*-coated single-stranded DNA is introduced into recipient cells by conventional methods, such as chemical transformation or electroporation. In whole cell shuffling techniques, the fragments undergo homologous recombination with cognate endogenous genes. Because

of the increased frequency of recombination due to recombinase coating, the fragments need not be introduced as components of vectors.

Fragments are sometimes coated with other nucleic acid binding proteins that promote recombination, protect nucleic acids from degradation, or target nucleic acids to the nucleus. Examples of such proteins includes *Agrobacterium virE2* (Durrenberger et al., *Proc. Natl. Acad. Sci. USA* 86, 9154-9158 (1989)). Alternatively, recipient strains can be deficient in RecD activity. Single stranded ends can also be generated by 3'-5' exonuclease activity or restriction enzymes producing 5' overhangs.

Transducing Shuffled Nucleic Acids into Plants.

As noted herein, it is particularly desirable to transduce plants with shuffled nucleic acids to reduce the level of mycotoxins in the plants, and/or to practice the shuffling procedures in plant cells. Reduction of mycotoxins benefits the plants by making them resistant to mycotoxicosis, as well as be making the plants safer for consumption.

Methods of transducing plant cells with nucleic acids are generally available. In addition to Berger, Ausubel and Sambrook, useful general references for plant cell cloning, culture and regeneration include Jones (ed) (1995) Plant Gene Transfer and Expression Protocols-- Methods in Molecular Biology, Volume 49 Human Press Towata NJ; Payne et al. (1992) Plant Cell and Tissue Culture in Liquid Systems John Wiley & Sons, Inc. New York, NY (Payne); and Gamborg and Phillips (eds) (1995) Plant Cell, Tissue and Organ Culture: Fundamental Methods Springer Lab Manual, Springer-Verlag (Berlin Heidelberg New York) (Gamborg). A variety of Cell culture media are described in Atlas and Parks (eds) The Handbook of Microbiological Media (1993) CRC Press, Boca Raton, FL (Atlas). Additional information for plant cell culture is found in available commercial literature such as the Life Science Research Cell Culture Catalogue (1998) from Sigma- Aldrich, Inc (St Louis, MO) (Sigma-LSRCCC) and, e.g., the Plant Culture Catalogue and supplement (1997) also from Sigma-Aldrich, Inc (St Louis, MO) (Sigma-PCCS). Additional details regarding plant cell culture are found in R.R.D.Croy, Ed. Plant Molecular Biolgy (1993) Bios Scientific Publishers, Oxford, U.K.

The nucleic acid constructs of the invention are introduced into plant cells,

5 either in culture or in the organs of a plant by a variety of conventional techniques. For  
example, the DNA construct can be introduced directly into the genomic DNA of the  
10 plant cell using techniques such as electroporation and microinjection of plant cell  
protoplasts, or the DNA constructs can be introduced directly to plant cells using ballistic  
5 methods, such as DNA particle bombardment. Alternatively, the DNA constructs are  
combined with suitable T-DNA flanking regions and introduced into a conventional  
15 *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium*  
*tumefaciens* host directs the insertion of the construct and adjacent marker into the plant  
cell DNA when the cell is infected by the bacteria.

10 Microinjection techniques are known in the art and well described in the  
scientific and patent literature. For example, a number of methods are described in Jones  
20 (ed) (1995) Plant Gene Transfer and Expression Protocols-- Methods in Molecular  
Biology, Volume 49 Human Press Towata NJ, as well as in the other references noted  
herein and available in the literature.

15 For example, the introduction of DNA constructs using polyethylene  
glycol precipitation is described in Paszkowski, *et al.*, EMBO J. 3:2717 (1984).  
Electroporation techniques are described in Fromm, *et al.*, Proc. Nat'l. Acad. Sci. USA  
30 82:5824 (1985). Ballistic transformation techniques are described in Klein, *et al.*, Nature  
327:70-73 (1987). Additional details are found in Jones (1995) *supra*.

20 *Agrobacterium tumefaciens*-mediated transformation techniques, including  
disarming and use of binary vectors, are also well described in the scientific literature.  
35 See, for example Horsch, *et al.*, Science 233:496-498 (1984), and Fraley, *et al.*, Proc.  
Nat'l. Acad. Sci. USA 80:4803 (1983). *Agrobacterium*-mediated transformation is a  
preferred method of transformation of both monocots and particularly dicots.

40 25 To use shuffled sequences, recombinant DNA vectors suitable for  
transformation of plant cells are prepared. A DNA sequence coding for the desired  
shuffled mycotoxin detoxification DNA is transduced into the plant. Where the sequence  
45 is expressed, the sequence is optionally combined with transcriptional and translational  
initiation regulatory sequences which further direct the transcription or translation of the  
30 sequence from shuffled the gene in the intended tissues of the transformed plant.

### Regeneration of Transgenic Plants

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans, et al., Protoplasts Isolation and Culture, Handbook of Plant Cell Culture, pp. 124-176, Macmillian Publishing Company, New York, (1983); and Binding, Regeneration of Plants, Plant Protoplasts, pp. 21-73, CRC Press, Boca Raton, (1985). Regeneration can also be obtained from plant callus, explants, somatic embryos (Dandekar, et al., J. Tissue Cult. Meth. 12:145 (1989); McGranahan, et al., Plant Cell Rep. 8:512 (1990)), organs, or parts thereof. Such regeneration techniques are described generally in Klee, et al., Ann. Rev. of Plant Phys. 38:467-486 (1987). Additional details are found in Payne (1992) and Jones (1995), *both supra*.

Preferred plants for expression of mycotoxin resistance genes include those for which mycotoxins are a significant problem, such as plants in the family *Graminae* (including corn, rye, triticale, barley, miller, rice, wheat, oats, etc.) plants in the family *Leguminosa* (e.g., peanuts, peas, beans, and the like) and nut plants (walnut, pecan, etc.) .

More generally, important commercial crops which benefit from mycotoxin detoxification include both monocots and dicots. Monocots such as plants in the grass family (*Gramineae*), such as plants in the sub families *Fetucoideae* and *Poacoideae*, which together include several hundred genera including plants in the genera *Agrostis*, *Phleum*, *Dactylis*, *Sorgum*, *Setaria*, *Zea* (e.g., corn), *Oryza* (e.g., rice), *Triticum* (e.g., wheat), *Secale* (e.g., rye), *Avena* (e.g., oats), *Hordeum* (e.g., barley), *Saccharum*, *Poa*, *Festuca*, *Stenotaphrum*, *Cynodon*, *Coix*, the *Olyreae*, *Phareae* and many others. As noted, plants in the family *Gramineae* are a particularly preferred target plants for the methods of the invention.

Additional preferred targets include other commercially important crops, e.g., from the families *Compositae* (the largest family of vascular plants, including at

5 least 1,000 genera, including important commercial crops such as sunflower), and  
Leguminosae or the "pea family," which includes several hundred genera, including  
many commercially valuable crops such as pea, beans, lentil, peanut, yam bean,  
10 cowpeas, velvet beans, soybean, clover, alfalfa, lupine, vetch, lotus, sweet clover,  
5 wisteria, and sweetpea.

Plants in the genera *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*,  
*Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manihot*, *Daucus*, *Brassica*, *Raphanus*,  
15 *Sinapis*, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersicon*, *Nicotiana*, *Solanum*,  
*Petunia*, *Digitalis*, *Majorana*, *Cichorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*,  
10 *Antirrhinum*, *Heterocallis*, *Nemesia*, *Pelargonium*, *Panicum*, *Pennisetum*, *Ranunculus*,  
*Senecio*, *Salpiglossis*, *Cucumis*, *Browaalia*, *Glycine*, *Lolium*, *Zea*, *Triticum*, *Sorghum*,  
20 *Malus*, and *Aplum* are targets for mycotoxin detoxification, as are plants in the genera  
*Agrostis*, *Phleum*, *Dactylis*, *Sorgum*, *Setaria*, *Zea*, *Oryza*, *Triticum*, *Secale*, *Avena*,  
*Hordeum*, *Saccharum*, *Poa*, *Festuca*, *Stenotaphrum*, *Cynodon*, *Coix*, *Olyrae*, *Phareae*,  
25 *Glycine*, *Pisum*, *Cicer*, *Phaseolus*, *Lens*, and *Arachis* are targets for acquiring mycotoxin  
15 detoxification activity.

Common crop plants which are targets for mycotoxin detoxification  
30 include corn, rice, triticale, rye, cotton, soybean, sorghum, wheat, oats, barley, millet,  
sunflower, canola, peas, beans, lentils, peanuts, yam beans, cowpeas, velvet beans,  
20 clover, alfalfa, lupine, vetch, lotus, sweet clover, wisteria, sweetpea and nut plants (e.g.,  
walnut, pecan, etc).

In construction of recombinant expression cassettes of the invention, a  
plant promoter fragment is optionally employed which directs expression of a nucleic  
acid in any or all tissues of a regenerated plant. Examples of constitutive promoters  
40 25 include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'-  
or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, and other  
transcription initiation regions from various plant genes known to those of skill.  
Alternatively, the plant promoter may direct expression of the polynucleotide of the  
45 invention in a specific tissue (tissue-specific promoters) or may be otherwise under more  
precise environmental control (inducible promoters). Examples of tissue-specific  
30 promoters under developmental control include promoters that initiate transcription only

in certain tissues, such as fruit, seeds, or flowers.

Any of a number of promoters which direct transcription in plant cells can be suitable. The promoter can be either constitutive or inducible. In addition to the promoters noted above, promoters of bacterial origin which operate in plants include the octopine synthase promoter, the nopaline synthase promoter and other promoters derived from native Ti plasmids. See, Herrera-Estrella *et al.* (1983), *Nature*, 303:209-213. Viral promoters include the 35S and 19S RNA promoters of cauliflower mosaic virus. See, Odell *et al.* (1985) *Nature*, 313:810-812. Other plant promoters include the ribulose-1,3-bisphosphate carboxylase small subunit promoter and the phaseolin promoter. The promoter sequence from the E8 gene and other genes may also be used. The isolation and sequence of the E8 promoter is described in detail in Deikman and Fischer, (1988) *EMBO J.* 7:3315- 3327. Many other promoters are in current use and can be coupled to a mycotoxin detoxification nucleic acid to direct expression of the nucleic acid.

If polypeptide expression is desired, a polyadenylation region at the 3'-end of the coding region is typically included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from, e.g., T-DNA.

The vector comprising the sequences (e.g., promoters or coding regions) from genes encoding expression products of the invention will typically comprise a nucleic acid subsequence which confers a selectable phenotype on plant cells. The vector comprising the sequence will typically comprise a marker gene which confers a selectable phenotype on plant cells. For example, the marker may encode biocide tolerance, particularly antibiotic tolerance, such as tolerance to kanamycin, G418, bleomycin, hygromycin, or herbicide tolerance, such as tolerance to chlorosulfuron, or phosphinothricin (the active ingredient in the herbicides bialaphos and Basta). For example, crop selectivity to specific herbicides can be conferred by engineering genes into crops which encode appropriate herbicide metabolizing enzymes from other organisms, such as microbes. See, Padgett *et al.* (1996) "New weed control opportunities: Development of soybeans with a Round UP Ready™ gene" In: Herbicide-Resistant Crops (Duke, ed.), pp 53-84, CRC Lewis Publishers, Boca Raton ("Padgett, 1996"); and Vasil (1996) "Phosphinothricin-resistant crops" In: Herbicide-Resistant

Crops (Duke, ed.), pp 85-91, CRC Lewis Publishers, Boca Raton) (Vasil, 1996).

Transgenic plants have been engineered to express a variety of herbicide tolerance/metabolizing genes, from a variety of organisms-- and the expression of these genes in concert with the mycotoxin detoxification nucleic acid can be used a selectable marker for the presence of a vector comprising the mycotoxin detoxification nucleic acid. For example, acetohydroxy acid synthase, which has been found to make plants which express this enzyme resistant to multiple types of herbicides, has been cloned into a variety of plants (*see, e.g.,* Hattori, J., et al. (1995) Mol. Gen. Genet. 246(4):419). Other genes that confer tolerance to herbicides include: a gene encoding a chimeric protein of rat cytochrome P4507A1 and yeast NADPH-cytochrome P450 oxidoreductase (Shiota, et al. (1994) Plant Physiol. 106(1):17, genes for glutathione reductase and superoxide dismutase (Aono, et al. (1995) Plant Cell Physiol. 36(8):1687, and genes for various phosphotransferases (Datta, et al. (1992) Plant Mol. Biol. 20(4):619. Similarly, crop selectivity can be conferred by altering the gene coding for an herbicide target site so that the altered protein is no longer inhibited by the herbicide (Padgett, 1996). Several such crops have been engineered with specific microbial enzymes for confer selectivity to specific herbicides (Vasil, 1996). A wide variety of expression cassettes are known and available.

One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

#### Computer System Elements

Software elements for manipulating strings of characters which correspond to mycotoxin detoxification nucleic acids can be used to direct synthesis of oligonucleotides relevant to shuffling of mycotoxin detoxification nucleic acids. Integrated systems comprising these and other useful features, e.g., one or more of: a digital computer with additional features such as high-throughput liquid control software, image analysis software, data interpretation software, a robotic liquid control armature for transferring solutions from a source to a destination (e.g., for manipulating selection assay solutions) operably linked to the digital computer, an input device (e.g., a computer keyboard) for

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In an additional aspect, the present invention provides kits embodying the methods and apparatus herein. Kits of the invention optionally comprise one or more of the following: (1) a shuffled component as described herein; (2) instructions for practicing the methods described herein, and/or for operating the selection procedure herein; (3) one or more mycotoxin assay component; (4) a container for holding mycotoxin detoxification nucleic acids or enzymes, other nucleic acids, transgenic plants, animals, cells, or the like and, (5) packaging materials.

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In a further aspect, the present invention provides for the use of any component or kit herein, for the practice of any method or assay herein, and/or for the use of any apparatus or kit to practice any assay or method herein.

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Accordingly, the disclosures and descriptions herein are intended to be illustrative, but not limiting, of the scope of the invention which is set forth in the following claims. One of skill will recognize many modifications which fall within the scope of the following claims. For example, all of the methods and compositions herein may be used in different combinations to achieve results selected by one of skill. *All publications and patent applications cited herein are incorporated by reference in their entirety for all purposes, as if each were specifically indicated to be incorporated by reference.*

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**WHAT IS CLAIMED IS:**

1                   1. A method of making a nucleic acid encoding a mycotoxin  
2 detoxification activity, the method comprising:  
3                   recombining a plurality of parental nucleic acids to produce one or more  
4 recombinant mycotoxin detoxification nucleic acid comprising a distinct or improved  
5 mycotoxin detoxification activity; and,  
6                   selecting the one or more recombinant mycotoxin detoxification nucleic acid for  
7 one or more encoded mycotoxin detoxification activity or,  
8                   selecting the one or more recombinant mycotoxin detoxification nucleic acid for  
9 enhanced or reduced encoded polypeptide expression or stability;  
10                  thereby producing a selected shuffled mycotoxin detoxification nucleic acid,  
11 which nucleic acid encodes a selected mycotoxin detoxification activity.

1                   2. The method of claim 1, wherein the mycotoxin detoxification activity is  
2 selected from: inactivation or modification of a polyketide, inactivation or modification of  
3 an aflatoxin, inactivation or modification of a sterigmatocystin, inactivation or  
4 modification of a trichothecene, and inactivation or modification of a fumonisin.

1                   3. The method of claim 1, wherein the one or more recombinant  
2 mycotoxin detoxification nucleic acid is selected by detecting a change in a physical  
3 property of one or more mycotoxin in the presence of a polypeptide encoded by the one  
4 or more mycotoxin detoxification nucleic acid, or by detecting cell growth or survival for a  
5 cell transduced with the one or more mycotoxin detoxification nucleic acid, which cell is  
6 cultured in the presence of the one or more mycotoxin.

1                   4. The method of claim 1, wherein at least one of the parental nucleic  
2 acids is the same as, or homologous to, a nucleic acid selected from: a monooxygenase, a  
3 P450, trichothecene-3-O-acetyltransferase, a 3-O-Methyltransferase, a glutathione S-  
4 transferase, an epoxide hydrolase, an isomerase, a macrolide-O-acetyltransferase, a 3-O-  
5 acetyltransferase, and a cis-diol producing monooxygenase which is specific for furan.

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1                   5. The method of claim 1, wherein the parental nucleic acids are  
2 homologous.

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1                   6. The method of claim 1, wherein at least one of the parental nucleic  
2 acids does not encode an anti-mycotoxin activity.

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3                   7. The method of claim 1, wherein the parental nucleic acid encodes a  
4 polypeptide or polypeptide subsequence selected from: a monooxygenase, a P450,  
5 trichothecene-3-O-acetyltransferase, a 3-O-Methyltransferase, a glutathione S-transferase,  
6 an epoxide hydrolase, an isomerase, a macrolide-O-acetyltransferase, a 3-O-  
7 acetyltransferase, and a cis-diol producing monooxygenase which is specific for furan.

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1                   8. The method of claim 1, wherein any of: the parental nucleic acids, the  
2 one or more recombinant monooxygenase nucleic acid, and the selected recombinant  
3 mycotoxin detoxification nucleic acid, is cloned into an expression vector.

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1                   9. A nucleic acid encoding a mycotoxin detoxification activity made by  
2 the method of claim 1.

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1                   10. The method of claim 1, wherein the plurality of parental nucleic acids  
2 are shuffled to produce a library of recombinant nucleic acids comprising one or more  
3 library member nucleic acid encoding one or more mycotoxin detoxification activity,  
4 which library is selected for one or more mycotoxin detoxification activity selected from:  
5 inactivation or modification of a polyketide, inactivation or modification of an aflatoxin,  
6 inactivation or modification of a sterigmatocystin, inactivation or modification of a  
7 trichothecene, and inactivation or modification of a fumonisin, an increased ability to  
8 chemically modify a mycotoxin, an increase in the range of mycotoxin substrates which a  
9 polypeptide encoded by the nucleic acid can modify, an increased expression level of a  
10 polypeptide encoded by the nucleic acid, a decrease in susceptibility of a polypeptide  
11 encoded by the nucleic acid to protease cleavage, a decrease in susceptibility of a  
12 polypeptide encoded by the nucleic acid to high or low pH levels, a decrease in

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13 susceptibility of the protein encoded by the nucleic acid to high or low temperatures, and  
14 a decrease in toxicity to a host cell of a polypeptide encoded by the selected nucleic acid.

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1 11. A library of recombinant nucleic acids comprising one or more  
2 monooxygenase activity made by the method of claim 10.

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1 12. The library of claim 10, wherein the library is a phage display  
2 library.

1 13. The method of claim 1, wherein the parental nucleic acids are  
20 2 shuffled in a plurality of cells, which cells are prokaryotes or eukaryotes.

1 14. The method of claim 1, wherein the parental nucleic acids are  
25 2 shuffled in a plurality of cells, which cells are plants, yeast, bacteria, or fungi.

1 15. The method of claim 1, wherein the parental nucleic acids are  
2 shuffled in a plurality of cells; the method optionally further comprising one or more of:

30 3 (a) recombining DNA from the plurality of cells that display mycotoxin  
4 detoxification activity with a library of DNA fragments, at least one of which undergoes  
5 recombination with a segment in a cellular DNA present in the cells to produce  
35 6 recombined cells, or recombining DNA between the plurality of cells that display  
7 mycotoxin detoxification activity to produce cells with modified mycotoxin detoxification  
8 activity;

9 (b) recombining and screening the recombined or modified cells to produce  
40 10 further recombined cells that have evolved additionally modified mycotoxin detoxification  
11 activity; and,

12 (c) repeating (a) or (b) until the further recombined cells have acquired a desired  
45 13 mycotoxin detoxification activity.

1 16. A further recombined cell having acquired a desired mycotoxin  
50 2 detoxification activity made by the method of claim 15.

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1 17. The method of claim 1, wherein the method further comprises:

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2 (a) recombining at least one distinct or improved recombinant nucleic acid with a  
3 further mycotoxin detoxification activity nucleic acid, which further nucleic acid is the  
4 same or different from one or more of the plurality of parental nucleic acids to produce a  
5 library of recombinant mycotoxin detoxification nucleic acids;

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6 (b) screening the library to identify at least one further distinct or improved  
7 recombinant mycotoxin detoxification nucleic acid that exhibits a further improvement or  
8 distinct property compared to the plurality of parental nucleic acids; and, optionally,

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9 (c) repeating (a) and (b) until the resulting further distinct or improved  
10 recombinant nucleic acid shows an additionally distinct or improved mycotoxin  
11 detoxification property.

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1 18. The method of claim 1, wherein the one or more recombinant  
2 mycotoxin detoxification nucleic acid is present in one or more bacterial, yeast, plant or  
3 fungal cells and the method comprises:

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4 pooling multiple separate mycotoxin detoxification nucleic acids;  
5 screening the resulting pooled mycotoxin detoxification nucleic acids to identify  
6 distinct or improved recombinant mycotoxin detoxification nucleic acids that exhibit  
7 distinct or improved mycotoxin resistance activity compared to a non-recombinant  
8 mycotoxin resistance activity nucleic acid; and,

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9 cloning the distinct or improved recombinant nucleic acid.

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1 19. The method of claim 1, further comprising transducing the distinct or  
2 improved nucleic acid into a prokaryote or eukaryote.

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1 20. The method of claim 1, wherein recombining the plurality of parental  
2 nucleic acids is performed by family gene shuffling.

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1 21. The method of claim 1, wherein recombining the plurality of parental  
2 nucleic acids comprises individual gene shuffling.

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1                   22. A plant transduced with the mycotoxin detoxification nucleic acid of  
2 claim 22.

1                   23. The plant of claim 22, wherein the plant is selected from the families  
2 *Gramineae*, *Composite*, and *Leguminosae*.

1                   24. The plant of claim 22, wherein the plant is selected from the genera:  
2 *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*,  
3 *Geranium*, *Manihot*, *Daucus*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Datura*,  
4 *Hyoscyamus*, *Lycopersicon*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*,  
5 *Cichorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Heterocallis*,  
6 *Nemesia*, *Pelargonium*, *Panicum*, *Pennisetum*, *Ranunculus*, *Senecio*, *Salpiglossis*,  
7 *Cucumis*, *Browaalia*, *Glycine*, *Lolium*, *Zea*, *Triticum*, *Sorghum*, *Malus*, *Apium*, *Agrostis*,  
8 *Phleum*, *Dactylis*, *Sorgum*, *Setaria*, *Zea*, *Oryza*, *Triticum*, *Secale*, *Avena*, *Hordeum*,  
9 *Saccharum*, *Poa*, *Festuca*, *Stenotaphrum*, *Cynodon*, *Coix*, *Olyraeae*, *Phareae*, *Glycine*,  
10 *Pisum*, *Cicer*, *Phaseolus*, *Lens*, and *Arachis*.

1                   25. The plant of claim 22, wherein the plant is selected from corn, rice,  
2 triticale, rye, cotton, soybean, sorghum, wheat, oats, barley, millet, sunflower, canola,  
3 peas, beans, lentils, peanuts, yam beans, cowpeas, velvet beans, clover, alfalfa, lupine,  
4 vetch, lotus, sweet clover, wisteria, sweetpea and a nut plant.

1                   26. The plant of claim 22, wherein the plant exhibits mycotoxin  
2 detoxification.

1                   27. A DNA shuffling mixture, comprising: at least three homologous  
2 DNAs, each of which is derived from a nucleic acid encoding a polypeptide or  
3 polypeptide fragment which encodes mycotoxin detoxification activity.

1                   28. The DNA shuffling mixture of claim 27, wherein the at least three

5 homologous DNAs are present in cell culture or *in vitro*.

1 29. A method of increasing mycotoxin detoxification of a cell,  
2 comprising: performing whole genome shuffling of a plurality of genomic nucleic acids  
3 in the cell and selecting for one or more mycotoxin detoxification activity.

1 30. The method of claim 29, wherein the genomic nucleic acids are from  
2 a species or strain different from the cell.

1 31. The method of claim 29, wherein the cell is of prokaryotic or  
2 eukaryotic origin.

1 32. The method of claim 29, wherein the mycotoxin detoxification  
2 activity to be selected is selected from: inactivation or modification of a polyketide,  
3 inactivation or modification of an aflatoxin, inactivation or modification of a  
4 sterigmatocystin, inactivation or modification of a trichothecene, and inactivation or  
5 modification of a fumonisin, an increased ability to chemically modify a mycotoxin, an  
6 increase in the range of mycotoxin substrates for the cell, an increased expression level of  
7 a mycotoxin detoxification polypeptide in the cell, a decrease in susceptibility of a  
8 mycotoxin detoxification polypeptide in the cell to protease cleavage, a decrease in  
9 susceptibility of a mycotoxin detoxification polypeptide in the cell to high or low pH  
10 levels, a decrease in susceptibility of the protein in the cell to high or low temperatures,  
11 and a decrease in toxicity to the cell by a mycotoxin detoxification polypeptide expressed  
12 in the cell.

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